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THE PROBLEM OF AUTO-ANTIBODIES*

An Approach to a Theory

PIERRE GRABAR†

It is my aim today, not to enumerate facts that most of you already know, but rather to provoke a discussion about the explanation of these facts. I should like to propose some hypotheses and to listen to your opinions about them. Perhaps I should apologise in advance for subjecting you to a recital of hypotheses, but in my opinion, even a wrong hypothesis may be useful if it provokes an experimental approach to a problem; that is, the hypothesis, based on fundamental knowledge, may serve a useful purpose as a working hypothesis.

In recent years, the term "auto-antibody" is being used more and more, and many pathological phenomena are explained by the statement: "It is due to an auto-antibody." Therefore, it seemed to me that it would be of value to try to approach this problem from the point of view of classical immunology.

An auto-antibody, by definition, is an antibody formed by an organism against an antigen which exists in the same organism, *i.e.*, to an endogenous substance. This is in complete contradiction to the concepts of classical immunology: that an antibody is a substance which appears in an animal as a consequence of a parenteral introduction of a foreign antigen.

At this point, I ask your permission to open a parenthesis and to present, as suggested by Dr. Nowinski, a summary of the modern view of the formation of antibodies in general. This may facilitate the understanding of the problem of auto-antibodies.

It must be admitted, I think, that at least two, and possibly more, categories of cells participate in the elaboration of antibodies. It is known that microbes or particles are phagocytozed and in some cases, at least, digested. Then the various constituent

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* This text was presented at a Seminar in the Department of Bacteriology, The University of Texas, Medical Branch, Galveston, Texas, and makes no pretension of completeness.

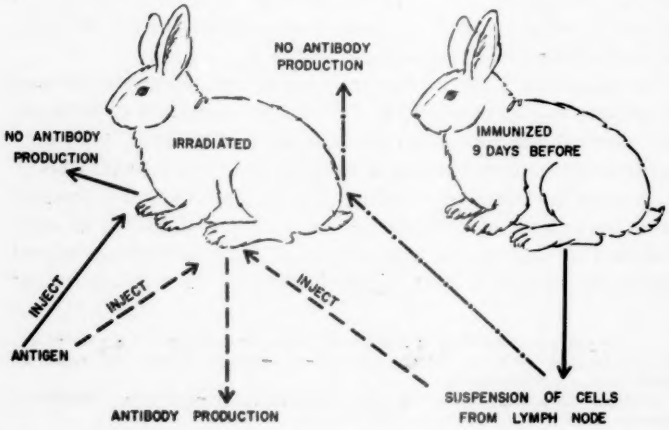
† Senior Research Fellow of the McLaughlin Foundation. Permanent address: Institut Pasteur, Paris (15), France.

antigens follow the same lines, probably, as do individual antigenic molecules. We know that in the case of a protein molecule, the first step in its degradation, by the action of a protease from reticulo-endothelial cells, is the formation of relatively large and still antigenic molecules.

We know, through the work of cytologists, using time-lapse cinematography, that soluble substances may enter the cells of the reticulo-endothelial system by *pinocytosis*. We may suppose that in the cells these substances are degraded, but that those which are antigenic provoke modifications of the template system of protein synthesis; *i.e.*, the deoxyribose nucleic acid.

We know now that at least one of the categories of white cells, the *plasmocytes*, may synthesise the antibodies, although it is possible that some other cells may do it also. However, plasmocytes alone are not capable of synthesising antibodies: it is probable that the lymphocytes also play a role in this process. I must add that it seems not impossible that lymphocytes and plasmocytes have a common origin and that their morphologic differences are a result of different physiological activities.

It has been shown, mainly by Dixon and co-workers, that if we inject a rabbit which has been irradiated (and is, therefore, incapable of synthesising antibodies upon injection of an antigen) with a suspension of lymphocytes from another rabbit,



immunized more than nine days before,[1] the receiver rabbit will produce antibodies, but only if he also receives an injection of the antigen. The irradiated rabbit has plasma cells but no lymphocytes. We can, therefore, postulate that the lymphocytes produce something which induces the synthesis of antibodies by the plasma cells.

It has been shown quite recently by Hamilton that the survival time of nucleic acids in leukemic leucocytes is longer (85 days) than is the normal life span of a lymphocyte. On the other hand, for many years it has been observed that lymphocytes are phagocytosed by reticular cells. As a result of this phagocytosis, there is a transfer of nucleic acids.

Thus, it seems possible to imagine the following chain of events: the lymphocytes form or contain the specific pattern of deoxyribonucleic acids for the synthesis of an antibody. These acids are transferred to the reticular cells which give rise to plasma cells in which the synthesis of antibodies occurs. Thus the whole cycle seems to be located in cells of mesodermal origin. I shall use this fact in my discussion. Now let us close the parenthesis.

For half a century, immunology has accepted the famous expression of Ehrlich of "Horror autotoxicus," meaning that an organism will not react against the introduction of his own constituents. But also about half a century ago, Metalnikoff showed that an animal can be immunized with its own spermatozoa. The immune serum produced in this way has been called cyto-toxic because it destroys cells; since that time many investigators have studied cyto-toxic sera made by injecting animals with different constituents of various organs.

At this point we must distinguish between various kinds of cyto-toxic sera. In most cases the sera have been prepared by the injection of heterologous organs, that is, organs from animals of another species; or from another animal of the same species, in which case the injected material is iso-antigenic. The antibody will be:

1) a hetero-antibody, which can be specific for an organ, for a species, for a group of animals of the same species (like

[1] These cells alone do not provoke the appearance of antibodies in the receiver-rabbit.

the specific substances of erythrocytes), and, possibly, for an individual.

2) an iso-antibody, which can be specific for an organ, for a group of individuals, or for an individual.
3) an auto-antibody, with a specificity limited to a particular organ, or better, to one or more constituents of an organ.

It is certainly very easy to produce hetero-antibodies, and when such hetero-antibodies are introduced into the animal against which they have been formed, it has been shown (for example, by Pressmann, by Anigstein, and by others using isotopes) that these antibodies will be fixed mainly—but not solely—on the corresponding organ. On the other hand, cytological examination of such organs shows that in many cases, there is an appearance of lesions, often strikingly similar to those which occur naturally. I am not competent to judge if such lesions are identical or only analogous to those seen in pathology, but I think that studies, using living cells in culture, should provide many, in this respect, interesting observations, because such studies can allow more precise and detailed observation of the mechanisms involved in such situations.

At any rate, because of the similarity of lesions, it has been suggested that in certain diseases of man and animals, the underlying pathological process may be the formation of antibodies, and such diseases have been called "auto-allergic."

Here again, we must make a distinction between lesions that occur in the case of classical allergy, and those produced by "auto-allergic diseases." In the first case, that is, in an allergy, the subject is sensitized to an exogenous substance, and the intervention of this substance may produce lesions. In the second case, the sensitising agent must be endogenous.

The other day, in a preceeding seminar, I mentioned an example, which is easy to understand and can be reproduced *in vitro*. We know that erythrocytes easily fix on their surface a polysaccharide from tubercle bacilli. If we add to a suspension of such sensitised red blood cells a serum which contains the antibody corresponding to this polysaccharide, we observe an agglutination of the erythrocytes. If we further add the complement, we obtain hemolysis. This is the principle of the so-called hemagglutination and hemolysis reactions of Middlebroock and Dubos. Some time ago, Stephen Boyden, working in my labora-

tory, observed that if polysaccharide from tubercle bacilli is injected into guinea-pigs, the cells of this animal are sensitized, even 48 hours later, *in vivo*. We may thus foresee that in an infected animal or man, such sensitisation may occur *in vivo*, and if the circulating blood contains antibody and complement, the cells should be lyzed. Such a mechanism may explain some anemias.

An analogous situation, of cellular lesions produced by an exogenous agent, occurs in the case of medicamentous allergy. Some drugs may be fixed by a cell constituent and if the individual has circulating antibodies towards this drug, the same mechanism may operate.

These cases cannot be classified as auto-allergy, because the active agent is not produced by the same organism. Let us return, then, to the real auto-immunization, that is, an immunization with the endogenous constituents of the same organism.

Since the end of the last century, many attempts have been made to immunize animals against organs, but in most cases these immunizations have been made either with organs from animals of different species or with organs from other animals of the same species. In the meantime, studies of blood transfusion have shown the existence of group specific substances, while studies on tissue grafts have demonstrated the existence of antigens specific for one particular individual (Medawar and Billingham). The first graft of skin persists some 8 to 20 days, while the second from the same donor is destroyed in a few days. If one injects a skin homogenate or even white cells, and then makes a graft of the skin from the same donor to the same recipient, this graft also persists for only a few days. Such experiments indicate the existence of individual antigens. Thus, genuine auto-immunization must be made with the organs or tissues of the same animal. Unequivocal experiments of this kind are relatively few and are not easy to perform. Let us take a few examples:

1) The oldest known, and probably the easiest to provoke, is the reaction of an animal to the injection of its own testis. In recent years, this has been studied by Freund and his colleagues in the U. S., and by Delaunay and Voisin, in France. The injections are made with Freund's adjuvent and provoke aspermatogenesis.

2) Another case is the production of lesions in the lens of the

eye, which resemble human phaco-anaphylactic endophthalmitis. We know also that if some material of the lens is left in an eye during an operation, the lens of the other eye may be severely affected. This can be explained only by the formation of anti-lens antibodies, reacting with the remaining lens.

3) A great number of publications have been devoted to the so-called "experimental allergic encephalomyelytis." The observed lesions resemble some human diseases.

This subject will be treated in the next Seminar by Prof. M. Minkowski. I shall, therefore, only mention that this disease has been produced in animals by the injection of brain tissue of any species, but also of portions of their own brain with adjuvants (Kabat and his co-workers).

4) Another disease, which is described as "experimental allergic neuritis," can be produced in animals by injecting material from the peripheral nervous system (Admas and B. Waksman).

5) For many years, many experimentators have tried to reproduce glomerulo-nephritis in animals. The easiest way to obtain lesions similar to those of man is to use the technique first described by Mazugi. It consists of immunizing an animal, for example, a duck, with the kidney of an animal from another species, say, a dog. If the immune-serum so produced is injected into a dog, lesions of glomerulo-nephritis are produced. This is a type of hetero-immunization, but some authors have been able to reproduce the same experiment with iso-kidneys, that is kidneys from the same species. On the other hand, so far, at least to my knowledge, genuine auto-immunization has not been achieved. Nevertheless, Vorlaender, in some human cases of nephritis, has recently observed the presence of antibodies which reacted to kidney extracts.

For more than two years, we have, with Miss Bourdon, tried to induce auto-immunization by injecting rabbits with one of their own kidneys. We have prepared homogenates, or autolysates (sterile conditions) of these kidneys and have injected them by various routes with or without adjuvants. So far we have not had any clear-cut positive results: we found neither anti-kidney antibodies, nor characteristic lesions. In order to avoid the absorption of the possibly existing circulating antibodies by the second, not extirpated, kidney, we have, after prolonged immunization, removed this second kidney and maintained the animals

for several days. Even with these precautions we were unable to find circulating auto-antibodies. This work is not completed, but we believe that direct and simple auto-immunization of a rabbit with his own kidney is not possible.

6) Recently, Witebsky and his collaborators have published a series of papers on the production of auto-antibodies against the thyroid. It seems that it is relatively easy to immunize an animal with the thyroid of another animal of the same species, but an animal can be immunized even by its own thyroid. Slight difficulties were encountered in the use of completely thyroidectomized animals, since these are in general poor antibody producers. In this work, circulating antibodies against thyroid have been observed, as well as specific lesions of the thyroid gland.[2]

So far, we have dealt only with those cases where direct immunization of an animal with its own material has been accomplished (auto-antigens). But, in many instances, similar lesions have been produced by immunization of an animal with some organs from another animal of the same species (iso-antigens), or even in those from a different species (hetero-antigens). In these immunized animals, the presence of antibodies reacting not only with the foreign antigens, but also with endogenous material, could be demonstrated. In explanation of this phenomenon, you may recall that the constituents of certain organs may be identical or nearly identical in different species; these are the so-called "organ-specific antigens." From experiments with hetero- or iso-immunization, it has been concluded that the animal reacted to the injection of foreign antigens by the formation of antibodies, and that these antibodies might then be capable of reaction with endogenous substances.

I shall cite a few examples of such experiments:

1) Voisin and Maurer have injected rabbits with a homogenate of rabbit skin; then, they grafted the same rabbits with skin from the same donor and this skin did not "take." But, later on, these rabbits developed a very particular type of generalized dermatitis, which could only be explained as a consequence of the formation of auto-antibodies against the animals' proper skin.

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[2] In a recent communication to the Biochemical Society, England, Roitt *et al.*, said that they observed, using the serum of patients suffering from lymphadenoid goitre, a specific precipitin reaction with an extract of thyrotoxic thyroid. They suggest that these sera contain an auto-antibody specific for a component of such thyroids.

2) Eyquem has injected into animals trypsinated erythrocytes from another animal. The injected animals developed antibodies which reacted not only with the injected erythrocytes, but also with their own erythrocytes, if these were treated by trypsin; *i.e.*, with an endogenous but modified substance.

This last example may perhaps be explained on the basis of a known immunological fact. We know that if we denature some endogenous proteins, for example, the serum proteins of the rabbit, and inject these into the same rabbit, the animal will form antibodies against these modified proteins. It has been concluded that modified proteins, *e.g.*, through denaturation, behave as exogenous antigens.

I. These considerations lead us to the first of the possible, hypothetical, explanations of the formation of so-called "auto-antibodies." This first explanation reconciles classical immunology with the problem of auto-antibodies and may be formulated in the following way: under certain conditions, for example, as a consequence of an infection, or a trauma, etc., some normal constituents of an organ or cells are transformed, modified, or denatured to such an extent that they become "foreign" to the organism, and the organism reacts by forming antibodies against them. Such a mechanism is absolutely "in the straight line" with the concepts of classical immunology and cannot, therefore, provoke objections. But the question arises, is such a mechanism sufficient to explain the observed pathological lesions? Two possibilities may be envisaged:

1) If the modification is persistent, that is, for example, if there is a latent infection, which provokes continuous codifications of normal constituents, then we may foresee a continuous formation of antibodies which react with these modified antigens and, ultimately, this reaction may induce lesions.

Some authors have envisaged the possibility that an antigen-antibody reaction as such may provoke transformations of normal constituents into modified antigens. Thus the process would continue. It seems to me that this last point is somewhat uncertain, because the antigen-antibody reaction itself does not produce modification of antigens, and some other mechanism must intervene to produce such a modification. If the antigen-antibody reaction provokes the action of the autolytic enzymes, then the

products should be those of "normal" degradation and not abnormally modified antigens.

However, if the modifications are permanent, then the mechanism is explained by the classical theory, even though the pathenogenesis of the lesions remains to be determined. In this case we are not permitted to speak of "auto-antibodies," because these antibodies are not directed against normal constituents of the organism, but are rather directed against a new antigen: an endogenous substance modified by an exogenous factor.

At this point, I may mention that some authors explain certain of these pathologic phenomena on a still more classical immunological basis. For example, speaking about glomerulo-nephritis, they propose the following explanation: in some infections, like those produced by streptococci, there is a formation of antibodies against bacterial constituents. It is assumed that these bacterial constituents may be firmly bound to some substances of the kidney. The reaction of the antibodies with those cell-combined bacterial antigens (or haptenes) may provoke lesions. This mechanism is certainly plausible, but if we accept it, we have yet to explain why such lesions may become chronic, unless we assume the presence of a chronic infection. If such a mechanism actually occurs, we should not speak about an "auto-antibody" nor an "auto-allergic disease." The principle of this mechanism is the same as in the case of medicamentous allergy or that of passive semolysis, as I cited earlier.

2) The alternative possibility, *i.e.*, that the modification of the normal constituents is only temporary, makes the explanation of the appearance of lesions still more hypothetical. Nevertheless, many authors have expressed the view that the antibody produced against the modified endogenous substance, may react not only with the modified antigen, but also with the normal endogenous substance, by a kind of cross-reaction.

As you know, a cross-reaction between an antibody and two different antigens is due to the existence in these two antigenic molecules of identical, or very similar chemical groupings. In the particular case which we are now discussing, if we assume that the antibodies against a modified substance may react with the natural substance, then we must also assume that part of the determinant specific group of the modified antigen corresponds to preexisting chemical groupings in the original substance. If

this actually occurs it would be a true auto-antibody, because such an antibody would react, even if only partially, with groupings existing in the native substance.

In this connection, I may mention some results which we have obtained a year ago with Dr. Marc Stahmann in my laboratory. We immunized rabbits with different synthetic polypeptides formed by polymerization of a single amino-acid, such as polyglutamic acid, or polylysine, or polyleucine. The sera of those rabbits gave specific precipitation reactions with the injected substance. But, unexpectedly, some of these antibodies, namely those against polylysine and polyglutamic acid, also reacted with several natural proteins. In other words, the grouping on the antibody molecule which reacted with the specific grouping on the synthetic polypeptide found a similar or identical grouping on some native protein molecules. I mention these facts because they may, perhaps, serve as a model for the cross-reactions of the type which I was discussing.

On the other hand, we know also that the structure of some microbial constituents resembles that of normal substances present in higher organisms (for example, hyaluronic acid or certain polysaccharides). In this case, an antibody to the bacterial antigen or hapten can give a cross-reaction with this normal constituent. But we do not yet know if the organism can develop antibodies to this particular grouping of the bacterial antigen with the same structure as its own constituent.

In summary of my first hypothesis, I may say that: in some circumstances, such as under the action of an infectious agent, a modification of certain constituents of the organism may provoke the formation of antibodies.

a) If this modification is permanent, or persistent, there is a formation of antibodies against this modified substance and the interaction of these may explain some of the lesions. However, this phenomenon would not be an "auto-allergy."

b) If the modification is not permanent, we must assume, in order to explain the presence of persistent antibodies or of persistent lesions, that the antibodies against the modified substance react with the native substance and that this reaction produces a new modification, which again provokes the formation of antibodies. Such a mechanism seems

to me somewhat complicated and perhaps this hypothesis should be modified.

II. The second hypothesis which I will now discuss, has a completely different starting point. It is based on the general theory of the production and the role of antibodies which I have proposed more than ten years ago. This theory does not take into account differences between endogenous and exogenous substances.

As you know, antibodies have the same chemical and physical properties as some of the normally occuring gamma-globulins. Some antibodies may have different properties than other antibodies, for example, they may have different molecular weights or different electrophoretic mobilities, but globulins possessing the same physical properties also exist in normal serum. If we consider the antigenic properties of antibodies, their specificity corresponds to the specificity of normal globulins, that is: if we prepare an anti-serum by injecting antibodies, this anti-serum will react against globulins from a normal serum, as well as with the injected material.

The site of synthesis of antibody and normal globulins also appears to be identical. The results obtained by various authors, and particularly by Good, on agammaglobulinemic patients, show strict parallelism between the absence of plasma cells, and the absence of both antibody and gamma globulin. Thus, the only difference between a globulin that we call an antibody and a globulin of the same properties present in normal serum is that we can distinguish an antibody by its specific reaction with an antigen. And this is also its distinctive characteristic; indeed, by definition, an antibody is a substance that fixes an antigen.

Actually, we do not know the precise physiological role played by many of the globulins present in normal serum. Some may be antibodies specific for the thousands of different antigenic substances that are encountered by any living organism: symbiotic microbes, and occasional penetration of other antigens through the skin, the respiratory or digestive tracts, etc. But more and more evidence has accumulated that the main role of serum proteins is to serve as carriers of various substances: lipids, carbohydrates, hormones, metals, etc. It has been shown by Redetzki and his co-workers, by ourselves with Uriel, and by Scheidegger and others, that if a man is given a large quantity of oil, there is, after a certain time, an increase in the lipo-proteins in the serum.

Two years ago, Dr. Kahn, speaking here, said, that if he injects some oil into a rabbit, his so-called "universal reaction," which is a lipid reaction, increases. We know also that some hormones, such as the estrogens or insulin, are transplanted in the serum in a complex with some particular globulins. We have found, using a very sensitive immunological reaction (passive haemagglutination of tanned red cells), that normal sera may react with such substances as gelatin, some other proteins, and glycogen, etc. It seems, therefore, permissable to conclude that the function of normal globulins is to fix (or combine with) certain substances and so transport them to the site where they are used. In this way, the globulins play a role as regulators of physiological mechanisms. In some cases it has also been shown, that this fixation is specific, that is, that certain globulins are specialized in the transport of certain definite substances.

If we accept the idea that the normal role of the proteins of the serum is to serve as carriers of some metabolic or other products, and that such combinations are more or less specific, then the difference between the functions of these globulins and the role of antibodies vanishes. We may then say, that the antibodies, like the globulins of any normal serum, are carriers (or fixators) of some substances. Certainly antibodies are considered more specific, but even this specificity may not be absolute because more and more immunological cross-reactions are being cited in the literature.

In short, my theory is to consider the formation of antibodies not as a special defense mechanism, but as a particular case of a general physiological mechanism of formation of globulins adapted to fix, and ultimately to carry, a definite substance.

If we now return to the question of auto-antibodies, it seems to me that this phenomenon can be considered as a particular manifestation of this general mechanism. I mentioned the transport by normal globulins of metabolic products; such transport can be extended to the degradation products of cells or tissues, which are constantly formed in a living organism.

We can thus conceive the possibility that in cases of massive destruction or degradation of certain tissues or of certain cells, as a result of infection or trauma, the organism may react by increased synthesis of globulins capable of fixing the degradation products.

If our hypothesis is correct, that is, if the role of normal globulins is to fix the products of cellular metabolism or degradation, the globulins which we call auto-antibodies would be only a pathological exaggeration of a normal physiological process. In other words, the auto-antibodies would be transporters of normal catabolic products and it is only their presence at a concentration sufficient to be detected by our present methods, that would be pathologic. More sensitive methods should detect them even in normal conditions.

III. The third hypothesis which I submit to your criticism is based on other facts.

We know, since the work of Medawar and of Billingham on animals, and of Hasek on eggs, that if an embryo has been in contact with certain antigens, the animal, after birth, will not form antibodies against these antigens. This phenomenon has been called "acquired tolerance." In most cases the antigens used have been of related origin, for example, erythrocytes of a related species such as hen and pheasant, or white cells or skin of two different strains of mice, etc. Different techniques have been used: injection in the embryo in utero, or symbiosis of two eggs, or injections into the new-born during the first days of life. In general, it seems that prolonged acquired tolerance can be obtained by these techniques only for antigens from various strains or races of the same species. For antigens of different species the acquired tolerance is of a relatively short duration. But a recent publication of Simonsen seems to indicate that, at least in the case of eggs, a more prolonged tolerance can be obtained by injecting the eggs not on the 11th day of incubation (as usual), but rather on the 17th day.

These facts suggest a hypothesis that is somewhat nearer to the classical immunology than the preceeding one, which makes no particular differences between endogenous or exogenous substances.

In this case, we suggest a slight modification of the term endogenous by considering that only those substances that exist in the embryo before a certain stage of development are truly endogenous for this organism and are, therefore, incapable of provoking the formation of antibodies. All those substances whose synthesis starts after this stage of development or that are situated in the organism in such a position that they normally do not

come into contact with the sites of antibody production, would be considered exogenous by the organism, although they are normally present within it. If, for some reason, for example, pathological conditions such as trauma, etc., these substances come into contact with the sites of antibody production, they may stimulate the synthesis of antibodies. If this mechanism occurs, such antibodies would be true auto-antibodies.

It may be interesting to recall at this point, that as previously mentioned, the formation of antibodies is due to cells of mesodermal origin and all of the experimental auto-allergies are produced by substances originating from tissues of ectodermal, endodermal, or of possibly questionable origin. This has been also noted in a recent discussion by Byron Waksman.

The exact mechanism of acquired tolerance is not yet understood, but a tentative explanation may be based on enzymatic activity. We know that a dead tissue may undergo autolysis by means of enzymes capable of degrading the constituents of the cells. It is probable that these enzymes are synthesized at a definite stage of embryological life and that they are adapted to degrade those substances which exist at that stage of development. If, at this moment, we introduce a new substance, the cells still contain the potentiality of forming an enzymatic system capable of degrading this substance. But, in a later period, this capacity may be lost and the introduction or the synthesis of a new substance, which cannot be completely degraded, then induces the formation of antibodies.

Recent studies of my collaborator, Cl. Lapresle, have shown that an antigen, such as human serum albumin, is attacked by a protease (pH opt=3.0–3.5) from the reticuloendothelial cells of a rabbit; this antigen is first broken down to relatively large products which correspond to the various antibodies produced by the rabbit against the serum albumin. Preliminary experiments show that at least these first degradation products are still antigenic. It seems then that it is not necessarily the original size of a molecule that determines its antigenicity, but rather its inability to undergo complete degradation by intracellular enzymes. On the other hand, the "own molecules" may undergo complete degradation, and are, therefore, not antigenic. In this case, when I say "own molecules," I mean the molecules which are synthesised before a certain stage of development.

In a recent publication, Bauer, Peckham, and Osler, report that they were unable to obtain "tolerance" in rats for sheep erythrocytes and contend that most positive experiments of this sort were obtained when large amounts of antigen (or living cells which could multiply) were injected into the embryos. They compare this situation to that of "immunologic paralysis" and suggest that these phenomena may simply be a consequence of overwhelming quantities of antigens. However, this would not solve the problem because we do not know much more about the mechanism of "immunologic paralysis" than about that of "acquired tolerance." If it were true that it is only the large quantities of antigen that are responsible for the absence of antibody formation, there should be a period, when those quantities would be sufficiently diminished to correspond to a level when antibody production could occur. (The mean half lifetime of a normal protein molecule in the body generally does not exceed some 15–20 days.[3]) In an earlier publication Cinader and Dubert point out that their antigen (human serum albumin, HSA) contained two impurities (alpha-globulins) and although the quantity of HSA used for the injection into new-born rabbits was large, the quantity of the injected impurities was small. Nevertheless, the animals produced antibodies neither to the HSA, nor to the impurities. Finally, as has already been said, it seems that "acquired tolerance" is more effective in cases where the antigenic substance is of related origin, and sheep erythrocytes may be too unrelated to the rat.

In summary, the three theories which I have developed can be condensed into a few words:

1) Possibility of formation of antibodies against a modified endogenous substance. These antibodies could perhaps also react with the native substance, and thus provoke lesions.

2) The detectable antibodies against a normal endogenous substance may be only a pathological exaggeration of a normal physiological process, the formation of globulins, whose function it is to fix and carry products of metabolism or degradation.

3) Substances that are synthesised in the organism only after a certain stage of embryological development or which are situ-

[3] The case of pneumococcal or other bacterial polysaccharides is somewhat special, because they may persist in the mammalian organisms for long periods of time, since they probably have no enzymes capable of degrading these substances.

ated in such a way that normally they do not come into contact with the sites of antibody production (local barriers or different embryological origin of tissues), may act as antigens and provoke the formation of auto-antibodies; they behave in effect, as exogenous substances.

It is likely that no one of these explanations is entirely correct, and that in some cases one is nearer to the truth, and in some other cases, another would fit better. The weakness of the first hypothesis lies in the postulate that antibodies formed against a modified substance react with a native substance. If that can happen, this hypothesis may explain some of the pathological phenomena under discussion. The second hypothesis may encounter difficulties in the explanation of how an increase in some particular globulins can induce a pathologic process. The third theory, perhaps with some additions or modifications, seems to fit the known facts more easily. However, as now formulated, it cannot be applied to all of those cases which are often considered "auto allergic;" namely to those cases where the lesions are found in tissues of mesodermal origin. To include them, a supplementary hypothesis must be formulated, namely that some of the constituents of such tissues do not appear until very late in embryological life, and that they normally have no contact with the sites of antibody formation.

I hope you will forgive me that a great part of this seminar was concerned with hypotheses, but I also hope that my discussion of these hypotheses will stimulate some new experimental approaches to this very important problem.[4]

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[4] The author takes this opportunity to express his gratefulness to Professor W. W. Nowinski and Dr. Rose Schneider for their help in correcting this manuscript, to Mrs. Margaret Cowser Earle for typing, and to the McLaughlin Foundation for its invitation.

INFLUENCE OF SEX HORMONES ON THE MANIFESTATIONS OF FAT-DEFICIENCY*

GARNER F. KLEIN, JOHN C. FINERTY,† THEODORE C. PANOS, AND RICHARD L. WALL

The manifestations of the fat-deficiency syndrome are more pronounced in male than in female rats (Burr and Burr, 1929; Panos and Finerty, 1954). Dermatitis appears earlier and is more severe. Complicating infections, particularly pulmonary, are more common and mortality rate is higher. The ultimate suppression of growth in males is greater.

The early observations of Burr and Burr (1930) on fat-deficient rats revealed striking disturbances of sexual function in both sexes. In females, these consisted of delayed ovulation, impairment of reproductive ability, and inability to nourish their young. In males, there was a decrease of sexual interest and in most cases complete sterility. These findings led the authors to suggest an inability of the fat-deficient animals to synthesize the sex hormones. Since that time, further observations have included atrophy of testes and male accessory organs (Evans *et al.*, 1935; Greenberg and Ershoff, 1951), faulty ovarian, uterine and placental function (Maeder, 1937), and various other endocrine changes (Panos and Finerty, 1953, 1954), which further relate fat-deficiency to hormonal disorder.

In an attempt to explore further the relationship between fat-deficiency and the sex hormones, the following experiments were undertaken.

Methods

Weanling albino rats of the Holtzman strain weighing 45–50 gm. were obtained for these experiments. Sixty males and 64 females were placed on a fat-free diet, the composition of which is given below, while 25 males were maintained on laboratory chow.[1]

Of the 64 females, 16 were kept intact, and 48 were ovariectomized; 16 of the latter were given daily estrogen injections, 16 were given daily androgen

* Departments of Anatomy and Pediatrics, The University of Texas Medical Branch, Galveston. Supported in part by grant A-380, National Institutes of Health, USPHS. Received for publication October 13, 1956.

† Present address: University of Miami School of Medicine, Coral Gables, Florida.

[1] Purina.

injections, and 16 were maintained ovariectomized and uninjected. The estrogen used was Estrone (Lilly), and it was administered subcutaneously in amounts of 2 gamma daily. The androgen given was Testosterone USP (Ayerst) and was similarly administered, dosage being 100 gamma daily. These levels of dosage were determined to represent physiological levels. Both materials were in aqueous suspensions to avoid introduction of any fat substances. The animals were maintained for nine weeks in this manner. During this period, they were weighed weekly, at which time each was inspected for the development of tail lesions. These lesions are the first manifestation of dermatitis so characteristic of fat-deficiency and are used as an objective criterion for the development of the syndrome. The first signs are scaliness and ridging of the tail, following which a papular eruption appears at the base of the tail. This progresses to general involvement of the tail, with the papules becoming ulcerated and fissured, and finally in many instances, the tip of the tail becomes necrotic and sloughs.

At weekly intervals, the basal oxygen consumption rates of eight animals from each experimental group were determined. This was done by means of a closed circuit respirometer similar to that described by Holtkamp *et al.* (1955). Increased basal oxygen consumption is a fundamental and consistent change in fat-deficiency. It has been demonstrated by this laboratory as early as the second week on the fat-free diet and is perhaps the earliest manifestation of fat-deficiency (Panos and Finerty, 1954).

The composition of the fat-free diet is as follows: Fat-free, vitamin-free casein 18 per cent, sucrose 76 per cent, salts 4 per cent, cellulose 2 per cent. Vitamins were added in the following amounts per kilogram of feed: thiamine chloride 5 mg., riboflavin 10 mg., pyridoxine 5 mg., calcium pantothenate 50 mg., nicotinic acid 25 mg., para-aminobenzoic acid 10 mg., biotin 2 mg., inositol 1000 mg., folic acid 2 mg., B_{12} mannitol trituration 1.8 mg., menadione 50 mg., crystalline vitamin A acetate 2 mg., crystalline vitamin D_3 20 mg., and alpha-tocopherol 100 mg. The last three "fat-soluble" vitamins were added after being dissolved in absolute ethyl alcohol.

At the conclusion of the experiment, the animals were sacrificed, and sections of the following organs were taken for routine histological study: liver, heart, kidneys, skin, thyroid, and adrenals.

Results

Growth. The effects of fat-deficiency on growth as determined by weekly body weight measurement are shown in figure I. Typical suppression is apparent on comparing the growth of fat-free males to that of the chow males. Weight differences appeared at the end of the second week, and became progressively greater thereafter. This growth curve is relatively short, but reveals the early tendency of fat-deficient animals to reach a plateau.

The growth effects exerted by the various hormones are also shown in figure I. The ovariectomized female controls, and those

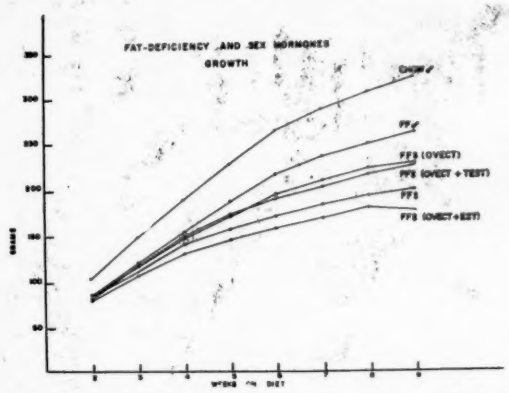

FIG. 1. Effects of diet and sex hormones on growth as expressed by body weight.

TABLE 1

FAT-DEFICIENCY AND SEX HORMONES
EFFECTS ON DEVELOPMENT OF TAIL LESIONS

% OF ANIMALS SHOWING TAIL LESIONS

WEEKS ON DIET	Chow ♂ (25)	FF ♀ (16)	FF ♂ (60)	FF ♀ (16) ovariectomized	FF ♀ (16) ovariectomized +testosterone	FF ♀ (16) ovariectomized +estrogen
4	0	0	0	0	38%	69%
5	0	0	9%	12%	44%	94%
6	0	0	18%	25%	80%	100%

TABLE 1. Effects of diet and sex hormones on development of tail lesions.

supplemented with testosterone have the highest growth rates, followed by the intact females. The ovariectomized estrogen injected group has the lowest growth rate. The rates of all female groups are lower than the males.

Lesions. As the experiment progressed, observations for tail lesions revealed their occurrence first in the ovariectomized group injected with estrogen. Next, the testosterone injected group developed lesions. The percentages of animals which developed lesions in each group are shown in table 1. The severity of the lesions was roughly proportional to the percentage of animals affected. Thus, at the end of 6 weeks, every animal in the estrogen injected group had lesions, most of which had progressed to necrosis of the end of the tail. The relative severity of the lesions in

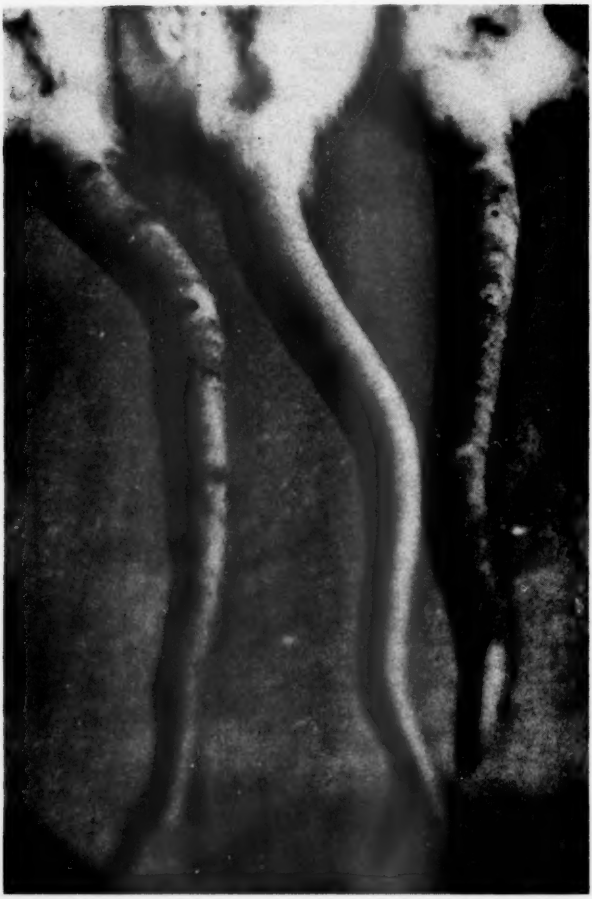

FIG. II. Effect of sex hormones on the severity of the tail lesions in fat-deficient female rats.
left—ovariectomy plus estrogen
middle—ovariectomy alone
right—ovariectomy plus testosterone

the hormone-injected groups at 5 weeks is shown in figure II. The ovariectomized females had a slightly higher incidence of leisons than the fat-deficient males, but the difference is probably insignificant, since the times of appearance were the same. The

chow-fed males and the intact fat-free females did not develop lesions at any time during the nine-week course of the experiment.

Oxygen Consumption. Basal oxygen consumption rates as illustrated in figure III showed the typical difference between fat-deficient and normal animals, as previously described. No differences due to sex or to injection with sex hormones were demonstrable.

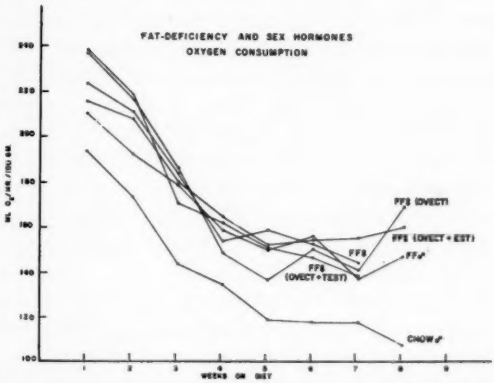

FIG. III. Effects of fat-deficiency and sex hormones on basal oxygen consumption. Basal metabolism drops with age in all groups but fat deficient animals maintain an elevated oxygen consumption.

Histology. Microscopic sections of the various organ tissues revealed changes consistent with the fat-deficiency condition as described previously (Panos and Finerty, 1953, 1954). Specifically, these included fatty infiltration of the liver, inter-tubular extravasation of blood in the kidneys, and thickening of the epidermis.

Discussion

Growth. The growth curves of both male groups are typical (Panos and Finerty, 1954). The growth of the females show patterns which can be explained on consideration of their various hormonal conditions. The ovariectomized uninjected and ovariectomized + testosterone groups have similar growth curves, which are higher than that of intact females. Increased body weight due to ovariectomy is a typical finding (Korenchevsky and Jones,

1946) and is usually associated with an increase of body fat (Korenchevsky, 1925; Stotsenberg, 1913), an observation which was apparent grossly at autopsy, even in fat-deficiency. The injections of testosterone did not alter the weight gain of ovariectomy, but its anabolic effects were apparent in that the body fat was not grossly increased as in ovariectomy alone.

The failure of ovariectomy to produce a weight increase when estrogen was injected correlates with the well-established fact that chronic estrogen administration slows body growth (Spencer, D'Amour, and Gustavson, 1932) through suppression of growth hormone production by the anterior pituitary (Reece and Leonard, 1939). The ultimate loss of weight, however, suggests more of a toxic or overdosage effect. A similar finding was described by Korenchevsky and Hall after ovariectomy (1937), but only at doses of 30 gamma of estrone per day. This indicates that fat-deficient animals may have a decreased tolerance for this hormone. There is some possibility that the apparent sensitivity may be related to the fatty infiltration of the liver noted in fat-deficient animals (Panos and Finerty, 1953, 1954). Impaired estrogen detoxification by nutritionally induced fatty livers has been observed in rats (Shipley and Gÿorgy, 1944). The finding of fatty livers in the present experiment suggests this possibility as an explanation for the decreased estrogen tolerance. The high levels of estrogen which could accumulate would cause growth effects similar to those caused by overdosage of the hormone.

Skin Changes. The requirements for essential fatty acids are approximately 5 times greater for male than for female rats (Greenberg, Calbert, Savage, and Deuel, 1950). This is probably related to the greater growth potential of male animals, due primarily to the anabolic effects of the male sex hormones. It is well known that signs of a deficiency of any element necessary for growth are most severe when the requirements for that element are greatest, *i.e.* when the growth rate is highest. For these reasons, the skin manifestations of fat-deficiency appear earlier and become more severe in males, whose growth rate is more rapid, than in females.

In addition, there is probably a protective effect against lesion formation exerted by estrogens in physiological amounts, through their direct stimulatory effect on keratinization and epithelial proliferation (Zuckerman, 1940).

The removal of the ovaries, of course, removes the source of estrogens and any protective effect they may exert, as mentioned above. There is, however, another effect of ovariectomy which tends to intensify the fat-deficiency. As shown by the weight curves, the ovariectomized animals are larger than intact females. This increased growth is a consistent finding (Korenchevsky and Jones, 1946) and whether it is due to an accumulation of fat or an actual structural increase, it serves to intensify fat-deficiency for the same reasons given earlier.

The fact that ovariectomized animals develop lesions as early as the intact males is probably due to a combination of the two factors mentioned, that is, the removal of estrogens and the increased body growth.

Both testosterone and estrogen exert anabolic effects (Paschkis, Rakoff, and Cantarow, 1954), the former to a much greater extent than the latter. The increase of anabolism by testosterone, as evidenced by the growth curves, is sufficient to explain the intensified fat-deficiency manifested by increased severity of skin lesions.

The stimulation of lesion formation by estrogen injections seems paradoxical in light of the previously proposed protective effect which estrogens seem to exert. The results however can be explained. There are three facts which clarify the picture. a) The levels of estrogens in fat-deficiency seem to be below normal. b) The levels of estrogen injected were designed to maintain physiological levels in an otherwise normal castrate. These two facts indicate that the injected estrogen levels were higher than those fat-deficiency. c) The skin is a sensitive end organ for estrogen stimulation, undergoing cellular proliferation, *i.e.* growth. With the increased estrogen levels, the anabolic activity of the skin increased, the fat-deficiency was intensified and manifested by more severe lesion formation.

It is felt that the higher estrogen levels, and the apparent toxicity of estrogens at the dosage given combined to produce additive effects of intensifying fat-deficiency and promoting lesion formation to a greater degree than did testosterone.

Oxygen Consumption. The evidence presented indicates that basal oxygen consumption is unaffected by sex hormones, either endogenous or exogenous. Although it is true that testosterone typically raises BMR, while estrogens do not alter it (Paschkis,

Rakoff, and Cantarow, 1954), the difference is obscured by the high degree of elevation resulting from fat-deficiency. Oxygen consumption increase, therefore, represents a fundamental metabolic derangement not associated with changes in sex hormone metabolism.

Summary

1. The effects of fat-deficiency as manifested by lesion formation are more severe in male than in female rats.

2. Ovariectomy stimulates body growth, thereby intensifying fat-deficiency and increasing lesion formation.

3. Androgens injected into ovariectomized females also stimulates growth and lesion formation.

4. Estrogens at higher than normal levels intensify fat-deficiency and increase lesion development. Fat-deficient females exhibit an intolerance to estrogens as manifested by growth suppression followed by weight loss, as well as severe lesion development.

5. The increased basal oxygen consumption rate of fat-deficiency is not modified by sex nor by injections of the sex hormones.

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THE ECOLOGY OF SUPERFICIAL FUNGUS INFECTIONS IN GALVESTON, TEXAS

A FIVE YEAR SURVEY*

MARY E. PINKERTON, J. FRED MULLINS, AND
EDWARD M. SHAPIRO

Records of the incidence of superficial fungus infections in various geographical areas of the world are fairly common in the literature. These studies are of great interest clinically as well as scientifically. It becomes apparent that there is a suggestive correlation of certain fungus species and their association with racial groups, and to a lesser extent with geographical locations; which is, perhaps, largely a function of the predominant nationality of the region.

Inasmuch as there has never been an extensive survey of the relative incidence of the various superficial dematophytes in Galveston, and because we had such data available to us, it was decided to make such a study. This region is of a special interest in that three races are represented in substantial numbers in our clinics, namely: white, negro, and Latin-American (Mexican). The climate is also rather unique, being moist and with subtropical temperature ranges.

Materials and Methods

Cultural material was secured from patients attending the Dermatology Clinic of the John Sealy Hospital, from referral cases in other clinics and wards in the hospital, and from the private practice of three of the doctors on the staff.

In most cases a dermatomycosis was suspected clinically and substantiated by finding fungus elements in KOH mounts of infected material. Wood's light examinations were also made of tinea capitis cases. Original isolations were usually made on Petri plates on Littman's oxgall agar, with the exception of a several months' comparative study in which Sabouraud's cycloheximide agar was also utilized. Pathogens were transferred to Sabouraud's dextrose agar in small perfume bottles with screw caps to secure giant colonies. In some cases, vitamin-deficient organisms and those not displaying fruiting

* From the Department of Dermatology, (J. Fred Mullins, M.D., Chairman and Director), The University of Texas Medical Branch, Galveston, Texas.
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bodies were also studied on Sabouraud's thiamine agar (0.0/mg./cc) Sabouraud's broth, cornmeal agar, brainheart infusion blood agar, yeast-morphology agar, chlamydospore agar, etc., were also occasionally utilized in identification of the fungi. Microscopic examinations of cultures were made in lacto-phenol cotton blue mounts.

Results

In the five calendar years, 1951 through 1955, 4,388 fungus cultures were made, mostly from cases of suspected dermatomycoses, with the isolation of pathogenic filamentous dermatophytes from 1,153 individuals. This number does not include subsequent repeat cultures nor those from diverse sites of the body in the same individual. The data were furnished by various workers in the Department of Dermatology. Records of yeast isolates were not included in this survey.

The total incidence per species on a yearly basis is shown in table 1, and the numerical occurrence of pathogenic fungi as regards species involved and sites of predilection is summarized in table 2. In tables 3 and 4 the data are compared regarding sex, race, age, and economic status for tinea capitis and tinea corporis respectively. The findings regarding race versus fungus species are tabulated in table 5. The incidences reported in various other parts of the world are listed in table 6.

The results of a detailed species analysis derived mostly from the data of the last twenty months of the survey follow:

(1) *Microsporum Audouini.* During the five-year period there were 40 cases of infection due to *Microsporum Audouini,* of which 36 were tinea capitis. During 1951, 3 cases were seen, and in 1952 none; whereas there were 14 in 1953, 10 in 1954, and 13 in 1955. In reviewing the data on these 40 patients the following facts were observed:

(a) *Sites:* Thirty-six had tinea capitis and 4 tinea corporis only. Of the former group 8 also had concomitant tinea corporis. These lesions occurred on the hands, arms, and trunk.

(b) *Sex:* There were 31 males and 9 females. Tinea capitis cases included 30 males and 6 females.

(c) *Race:* There were 10 whites, 30 negroes, and no Latin-Americans.

(d) *Age:* The age of those with tinea capitis ranged from 2 to 10 years, with the exception of one negro male of fourteen. The average age was 5.9 years. Those with only tinea corporis were from 2 to 49 years of age. The latter included a colored girl of 2, two colored females of 20 and 42, and a white male of 49.

(e) *Epidemiology:* Six pairs of siblings were involved: three pairs, a brother and sister; and 3 pairs, two brothers. Two pairs were whites

and 4 pairs were negroes. Of the adults, a 42-year-old negro woman, who had a circinate eczematous lesion of 1 cm. diameter on her left forearm, cared for a grandchild with tinea capitis due to *M. Audouini*. In regard to one negro child from a Catholic school in Galveston, the teacher reported that other children in the school were also apparently affected (1954).

(f) *Residence:* Twenty-five of the 40 resided in Galveston, and the remainder within a 50 mile radius; 2 being from the city of Houston. Of the 3 white patients living in Galveston, one was a child seen in 1953, and a child and an adult were seen in 1954.

(g) *Economic status:* Thirty-three were clinic patients and 7 private patients (6 whites and 1 negro).

(h) *Duration of infection:* Most of the patients had been infected for several months before coming for diagnosis and treatment. There was little history of follow-up on their charts, so presumably the treatment was effective, as was also indicated by the lack of evidence of spread of the disease. Treatment was conservative in all cases.

(i) *Clinical appearance:* The lesions were usually non-inflammatory, transient alopecia was generally present, and scaling was a common factor. A pustular follicular involvement was noted in two siblings from a near-by community (LaMarque), and a year later another negro child from the same area had a pseudo-kerion. Two negro children from Galveston showed kerion formation after treatment. The lesions were predominantly solitary or few. When dissemination occurred, the external ears and forehead were frequently involved. It was not possible to differentiate clinically the infections due to *Microsporum Audouini* and to *M. canis* as the latter was also sometimes non-inflammatory and responded poorly to treatment.

(j) *Cultures:* The eighteen cultures studied were quite similar in appearance on Littman's agar as well as on Sabouraud's. On the latter media the colonies were white, fluffy, and slow growing. At maturity there was a slight central boss, a powdery buff surface, and the reverse tended to become a dull buff brown. Radial striations were not noted.

Microscopically there were numerous chlamydospores, rare rudimentary fuseaux, and sometimes hyphae sporiferae.

The predominance of this species in the negro race in our area is interesting. We saw no cases in Latin-Americans during the five-year period.

(2) *Microsporum canis (lanosum).* In the five-year survey 124 patients had positive cultures for *Microsporum canis*, the yearly incidence being 21, 20, 30, 29, and 24 respectively. Ninety-five of

the 124 had tinea capitis. In the 41 cases studied in detail the following facts were noted:

(a) *Site:* Thirty-two had tinea capitis and 9 tinea corporis alone The latter were all whites. The association of tinea capitis and tinea corporis was not investigated. Lesions of the glabrous skin occurred on the shoulders, arms, chest, face and back.

(b) *Sex:* There were 22 males and 19 females. Of those with tinea capitis, there were 21 males and 11 females. All of the Latin-Americans were males, the negroes females, and there were 15 males and 8 females among the whites with tinea capitis.

(c) *Race:* There were 32 whites, 3 negroes, and 6 Latin-Americans. With tinea capitis there were 23 whites, 3 negroes, and 6 Latin-Americans. Tinea corporis alone was found only in the white race.

(d) *Age:* Those with tinea capitis ranged from one month to 10 years of age, except one who was 47 years old. The average age was 5.4 years. The 47-year-old patient was a negro woman diagnosed as having lupus erythematosus. No Wood's light examination was made, a subsequent culture was negative, and she was not seen thereafter. The 9 white persons with tinea corporis alone were aged 5, 6 (2), 10, 21 (3), 30, and 70. All were females except a 10-year-old male.

(e) *Epidemiology:* There were four sets of siblings, all whites and brothers and sisters, except one pair of sisters. Each pair resided in a different town. A number of parents of infected children reported similar lesions in playmates. There was no history of contact with infected animals.

(f) *Residence:* Most of these patients resided in Galveston or nearby towns, except one from Lubbock (West Texas).

(g) *Economic status:* Twenty were private and 19 clinic patients, besides 2 student nurses.

(h) *Duration of infection:* In most cases the infection was reported as of one to several months' duration.

(i) *Clinical appearance:* No specific data was secured regarding clinical appearance. However, we have noted that a number were non-inflammatory, some dandruff-like, and response to treatment was occasionally slow.

(j) *Cultures:* Most of the 41 cultures studied were typical, although there was some variation in the degree of reverse yellow pigmentation, some strains from tinea corporis lesions lacking it. On two different occasions cultures of infected hairs from a 7-year-old white male grew peculiar glabrous cerebriforme colonies on Littman's agar. Transfers to Sabouraud's medium were grossly similar to other cultures of *Microsporum canis*, but microscopically the

numerous fuseaux were narrower and more elongated than typical, and often contained 20 or more septae as opposed to the usual number of eight to thirteen. A 30-year-old white female with an arm lesion suspected infection from a pet monkey. Unfortunately, the monkey was unavailable for culture. Her culture differed slightly in gross morphology only.

Microsporum canis was most prevalent in the white race in this vicinity, although a few Latin-American and negro children were involved. A 3-year-old Latin-American male had two older brothers (5 and 10) with tinea capitis due to *Trichophyton tonsurans*. The other Latin-Americans were also males. All 3 of the negroes were females, of ages 4, 8 and 47. Both of the latter races showed only tinea capitis lesions.

(3) *Microsporum gypseum (fulvum)*. In the five-year survey there were 24 patients with *M. gypseum* infections, the annual incidence being 3, 7, 6, 5, and 3 respectively. The following data were secured regarding these 24 patients:

(a) *Site:* Eleven had tinea capitis only, and 13 had tinea corporis exclusively. The areas of distribution of the latter included axilla, legs, arms, chest, face, and external ears.

(b) *Sex:* There were 15 males and 9 females. Of those with tinea capitis, there were 7 males and 4 females.

(c) *Race:* There were 18 whites, 3 negroes, and 3 Latin-Americans. Seven whites, 3 negroes and one Latin-American had tinea capitis.

(d) *Age:* Those with tinea capitis were from 2 to 12 years of age. The average age was 5.8 years. The age of those with tinea corporis varied from two months to 62 years. Six were below the age of puberty: 2 months, 3, 4 (2), 10 and 11 years. The negroes were all males of 6, 7, and 12 years. One Latin-American male of 5 had tinea capitis; a 4-year-old male and 38-year-old female had tinea corporis.

(e) *Epidemiology:* There was no evidence of familial or general epidemicy.

(f) *Residence:* Thirteen of the 24 were from Galveston, 9 from neighboring small towns, and one each from Jasper and Bonchito, Oklahoma.

(g) *Economic status:* In addition to 10 clinic and 12 private patients, there was one medical student and one of the personnel (a Latin-American female telephone operator).

(h) *Duration of infection:* The majority of the patients had been infected for only several days or weeks before seeking treatment.

(i) *Clinical appearance:* In general the infections were extremely inflammatory; 5 with tinea capitis had kerions. One 7-year-old colored male showed a 3 x 5 cm. furuncle of one week's duration on

the scalp, with multiple elevated pustular spots and much pus and scab formation; but there was little pain or adenopathy. Another colored male, aged 6, also had a "carbuncle" on the right posterolateral side of the scalp with multiple sinuses but no pus; the lesion was not fluctuant, but there were cervical nodes. Although the hairs were not broken off in the carbuncle, the lesions healed with alopecia. The 2-month-old white male patient had a rash in the axilla from which *M. gypseum* was cultured. A third colored male of 12 had a scalp lesion near the ear of 2 days duration which resembled an endothrix infection, being dry and scaly; but there were no broken-off hairs. He also had pityriasis rosea. Most of the tinea circinata lesions were solitary and inflammatory, resembling Majocchi's granuloma in 2 cases. The 38-year-old Latin-American female telephone operator had an inflammatory lesion on the outer thigh. She had no pets nor contact with such, but she had spent a lot of time on the beach. A white female of 31 with a circinate patch of ten days duration in the axilla had puppies and cats at home. Another white female of 3 had a red macular eruption near her mouth of 2 days duration. A Latin-American male of 4 had a scaly erythematous plaque on the medial surface of the right leg persistant for several weeks.

(j) *Cultures:* The 8 cultures studied showed the characteristic cinnamon-colored granular surface, but the degree of reverse yellow pigmentation varied. The fuseaux were typical.

(4) *Trichophyton tonsurans:** There was a total of 253 patients in the survey with *Trichophyton tonsurans* infections, the yearly incidence being 67,[1] 55, 45, 35, and 51. One hundred ninety-two of these had tinea capitis. From data studied on 63 patients it was found:

(a) *Site:* Forty-four had tinea capitis and 19 tinea corporis only. Eight of the former also had tinea corporis. The regions of the glabrous skin included: neck, arms, chest, trunk, legs, nose, ear lobes, buttocks, hands, eyelid, and face.

(b) *Sex:* There were 35 males and 28 females. Twenty-nine males and 15 females had tinea capitis.

(c) *Race:* There were 7 whites, 29 negroes, 26 Latin-Americans and 1 Chinese. Those with tinea capitis included 4 whites, 21 negroes, and 19 Latin-Americans. The Chinese was 22 years old, a female (personnel) with a single lesion on the right thigh.

(d) *Age:* The ages of those with tinea capitis ranged from 2 to 13, with the exception of 3 Latin-American females aged 35, 41, and 62.

* Data were excluded from an extensive school survey that was made in 1951.

The average age of the children was 7.1 years. Tinea corporis involvement was found in individuals from 2 to 68 years of age.

(e) *Epidemiology:* In 7 instances there was involvement of 2 members of a family. There were 2 sets of negro brothers and one negro mother and son; all except the woman had tinea capitis. Among the Latin-Americans was a mother and son who showed tinea capitis due to *T. tonsurans* and tinea corporis due to *T. rubrum;* a brother and sister, the boy with tinea capitis and the girl with tinea corporis; two brothers with tinea capitis due to *T. tonsurans,* with a younger brother with a *M. canis* infection on the scalp; two other brothers with tinea capitis.

(f) *Residence:* A relatively small number were from Galveston, the majority from nearby small towns, and some of the Latin-Americans were from southwest Texas.

(g) *Duration of infection:* The scalp lesions were usually of relatively long duration, being mild and chronic. The infections of the glabrous skin were often of several months duration.

(h) *Economic status:* There were 52 clinic; 10 private patients and one personnel.

(i) *Clinical appearance:* This was a "black-dot" type of ringworm in the scalp, and was mostly non-inflammatory. However, very inflammatory, pustular, scalp lesions occurred occasionally, especially in pre-school negro children. Lesions of the glabrous skin showed some resemblance to those caused by *Trichophyton rubrum,* particularly the several cases of chronic generalized eruptions.

(j) *Cultures:* We have found a number of morphological types of colonies in *Trichophyton tonsurans,* and are currently making a study of the significance of these findings which will be published in a subsequent paper.

One Latin-American female of 5 having *Trichophyton tonsurans* infection of the scalp and glabrous skin, also had acute lymphatic leukemia. A 62-year-old Latin-American female had a generalized eruption of the scalp and glabrous skin, the latter resembling a *Trichophyton rubrum* infection. However, only *Trichophyton tonsurans* was cultured from all areas. Besides the mycosis of 16 years duration, she also had a serious cardiac condition. A 3-year-old Latin-American boy had a severe nutritional deficiency; another of 4 years had tinea capitis due to both *T. tonsurans* and *M. canis;* another of 3 years had a younger brother with a monilethrix condition of the hair, in addition to *T. tonsurans;* another Latin-American boy of 7 years with a lesion on the chin had a sister with a similar appearing lesion around one eye in which only scabies was found.

(5) *Trichophyton mentagrophytes:* In the survey *T. mentagro-*

phytes was isolated from 172 individuals, the annual incidence being: 45, 29, 31, 26, and 41 respectively. In a study of 54 cases the following facts were noted:

(a) *Site:* See table 2.

(b) *Sex:* Thirty-three were males and 21 females.

(c) *Race:* There were 35 whites, 10 negroes, and 9 Latin-Americans.

(d) *Age:* The ages of the patients with this infection ranged from 3 to 70, 24 of them being between 20 and 40.

(e) *Epidemiology:* Three student nurses, 2 medical students, 2 professional, and 3 other personnel were included in the 54 cases. The patients with t. pedis usually had acute flareups in the late spring of the year.

(f) *Residence:* Most of these patients resided in Galveston, although there were some from the cities of Houston, Dallas, and Port Arthur.

(g) *Economic status:* In addition to 19 clinic and 25 private patients there were 10 members of the personnel.

(h) *Duration of infection:* The cases seen were usually of recent duration and were acute.

(i) *Clinical appearance:* In the tinea pedis cases there were typically vesiculo-pustular lesions on the soles of the feet, and in t. corporis the lesions were usually inflammatory.

(j) *Cultures:* The cultures were mostly the fluffy type which became more or less powdery with age, white sometimes turning pinkish or buff, and remaining buff color on the reverse. The culture from a 4-year-old white female with an ectothrix infection of the scalp and face yielded a granular type of *T. mentagrophytes,* and similar cultures were secured from t. corporis inflammatory lesions. Several cultures were unusual and difficult to identify.

(6) *Trichophyton rubrum:* In the five-year survey, *T. rubrum* was isloated from 500 patients (43.4 per cent of all isolates), the annual incidence being 144, 94, 62, 75, and 125 respectively. Data on the detailed study of 176 patients follows:

(a) *Sites:* See table 2.

(b) *Sex:* One hundred and three were males and 73 females.

(c) *Race:* One hundred and four were whites, 44 negroes, and 28 Latin-Americans.

(d) *Age:* Ages ranged from 2½ months to 79, 101 being between 20 and 50, 4 were 10 or under and 10 were between 12 and 20 years of age. The latter included 5 males and 5 females; 5 whites, 2 colored, and 3 Latin-Americans. Six of the 10 had tinea pedis, and the other

tinea cruris, and 1 white male of 16 had lesions on hands and buttocks. Another of 10 had t. unguium.

(e) *Epidemiology:* Rather rarely had it come to our attention that several members of the same family have the infection. Lately we have seen a probable example in a colored man and wife both showing lesions in the groin from which *T. rubrum* was cultured. In another case a 6-year-old Latin-American boy and his mother both had generalized *T. rubrum* infections and t. capitis due to *T. tonsurans.* The number of generalized cases was noteworthy; in many instances they had been of long duration; and often the patients also had associated debilitating diseases. A 10-year-old white girl had lesions on her shoulders following several months of coma after an automobile accident.

(f) *Residence:* The majority lived in Galveston, some having come originally from Europe or South America. These patients often came to the clinic for other more acute conditions.

(g) *Economic status:* In addition to 102 clinic patients and 59 private patients there were 15 of the personnel; these latter suffered mostly from tinea pedis or tinea cruris.

(h) *Duration:* This mycosis was frequently of long duration, the patients having consulted numerous physicians with no substantial cures. A number had disseminated chronic infections which would occasionally become acute.

(i) *Clinical appearance:* The lesions were mostly non-inflammatory, solid, dry, scaly plaques of various configuration, of a dull red color. In some cases, they mimicked dermatoses of non-mycotic origin. There was frequent involvement of the buttocks.

(j) *Cultures:* Cultures were extremely variable, but the initial appearance on Littman's agar was quite distinctive except for the colors which varied from gray, yellow, green, orange to purple. In general, the organisms from the acute cases tended to produce more granular colonies with less superficial white down and more obvious purple basal growth. Some colonies also had radial furrows and even cerebriform wrinkling which approached the characteristics of *T. violaceum.* Fuseaux were frequent in the young colonies of the granular type but rare in the downy ones.

(7) *Epidermophyton floccosum: E. floccosum* was isolated from 33 patients in the 5-year study; the incidence being 11, 3, 4, 6, and 9 respectively. Details on the 33 patients follow:

(a) *Site:* See table 2.

(b) *Sex:* There were 23 males and 10 females.

(c) *Race:* There were 19 whites, 6 negroes, 5 Latin-Americans, and 3 regarding whom data was not available.

(d) *Age:* The age of 20 of these patients ranged from 6 months to 74, 8 being below 30 years of age.

(e) *Epidemiology:* As the number of cases was so small, no evidence of contagion was noted. There have been several medical students and doctors with this infection. Subsequent to this survey the organism has been isolated from a mother and son.

(f) *Residence:* Most of the patients were from Galveston.

(g) *Economic status:* Five of the 33 were personnel, 20 clinic and 8 private patients.

(h) *Duration of infection:* The cases of tinea pedis tended to be chronic with occasional flareups which responded readily to treatment . The tinea corporis infections were of more recent origin and in them an apparent cure was affected.

(i) *Clinical appearance:* Typical. Tinea pedis due to this infection was frequently associated with dyshidrosis and the lesions were boggy callouses. The t. corporis lesions were quite superficial.

(j) *Cultures:* Cultures have been typical. In several instances *T. rubrum* or *T. mentagrophytes* was also isolated from these patients.

(8) *Trichophyton verrucosum:* This organism was cultured from 2 patients. One was a 6-year-old white boy from LaMarque with a slightly inflammatory eruption on the trunk and scalp. The other was a white male of 26 from Jasper, Texas, who raised livestock. He presented an erythematous, elevated slightly tender, firm lesion 3 x 4 cm. with a small crusting ulceration in the center, located on the right mid-outer forearm. It had the appearance of a Majocchi's granuloma, was of 6 weeks' duration, beginning with pruritus and gradually becoming raised and red with a scaling edge. The cultures were isolated with difficulty on Littman's oxgall agar, and after frequent transfers grew fairly well on Sabouraud's dextrose agar. The colony grew to the size of a nickel, was irregularly heaped, glabrous to slightly downy, and there were irregular projections of submerged growth. The reverse was yellow. Microscopically there were moderate numbers of small elongate microconidia.

(9) *Trichophyton violaceum:* Three children of a Latin-American family living in Texas City and who had been born in Sugar Land (a small town near Houston) had t. capitis due to *Trichophyton violaceum.* They included a boy of 11 and his two sisters aged 14 and 16. The boy's infection arose spontaneously following trauma one and a half years previous to coming to the clinic. The present condition included a 2 x 2 cm. denuded, ulcerated area on the vertex of the scalp and a few scaly spots with occasional broken off hairs. The younger of the sisters reported "furunculosis" of the scalp 2 years previously, but at the time of examination showed only numerous

scaly patches on an erythematous base with scattered loss of hairs. The 16-year-old girl had follicular plugs in the scalp. Positive cultures were secured only on the former 2. The cultures were not seen by the authors.

(10) *Cladosporium Wernecki:* Two patients exhibited lesions typical of tinea nigra palmaris. One was a 4-year-old white male from Galveston, and the other a 17-year-old white female from Brazoria, Texas. The latter infection had been present for 6 years. The third patient was a 38-year-old colored female, with axillary lesions. The first cultures from the axilla yielded both black yeasty colonies which became filamentous and colonies which were immediately filamentous. From subsequent cultures taken after treatment, only the filamentous type of colonies developed.

Discussion

In tinea capitis a slightly lower incidence of Microspora than of Trichophyta has been found in Galveston (142 to 197). The predominance of Microspora in scalp infections has been usual in the U. S.,[1] Canada,[2] England,[3] Uruguay,[4] Argentina,[5] and Hawaii.[6] However, in Mexico[7] the incidence of Microspora was only 8.2 per cent, in France[8] 37 per cent; and the genus was not found at all in Jerusalem[9] nor in native children in India,[10] although it did occur in resident English children.

Microspora

Microsporum Audouini has been reported as predominant over *M. canis* in certain cities in the U. S.: Boston,[11] Manhattan,[12] Philadelphia,[13] Chicago,[14] Birmingham,[15] New Orleans,[16] St. Louis,[17] Indianapolis,[18] and Fort Worth.[19] However, other reports from these areas have been contradictory: in Boston,[20] *M. Audouini* accounted for only 25 per cent of the cases; in Manhattan (Lewis and Hopper) the 2 species occurred in equal ratios, also in Philadelphia (Weidman).[1] In England, *M. Audouini* was found to be of much higher incidence than *M. canis*, also in Paris in 1910. At present *M. Audouini* accounts for only 13 per cent of the Microspora in Paris.[21] The species was not reported in Mexico, Uruguay, or Brazil.[22]

Microsporum canis was stated to be more frequent than *M. Audouini* in the following areas: southeastern Tennessee,[23] Springfield, Mo. and Oklahoma City, San Antonio,[24] Austin, Texas,[25] Galveston,[26] and Phoenix.[27]

We can theorize as to an explanation of the distribution of *M. Audouini* in the U. S. It is undoubtedly endemic, but is tending to be kept from epidemic proportions by scientific methods of detection and control. The source of the data as regards economic status of the patients will also affect the relative incidence *i.e.*, clinic records will tend to show more *M. Audouini* than those of private practice. However, it is obvious that race and geography also play a role: *e.g.*, *M. Audouini* is unknown in Mexico and South America. The situation now appears to be that *M. Audouini* infections are pretty well kept under control in the U. S. with occasional outbursts especially in cities; but *M. canis* infections occur in substantial numbers all over the country, perhaps because there has been little effort to keep it down in the natural animal hosts. Negro children were found to be the most frequent victims of *M. Audouini* in our area, as was also the case in New Orleans. Perhaps this was largely attributable to economic status. On the other hand we saw no cases in Latin-Americans indicating probably that they were not susceptible.

Microsporum gypseum seems to occur throughout the U. S. as a minor cause of t. capitis, but in Galveston it was slightly more frequent in t. corporis (13 of 24 cases). We found that it represented 2.1 per cent of all tineas here, and 3.4 per cent in t. capitis. Sharp and Wegner[26] estimated 5 per cent in Galveston and commented on the seasonal incidence (summer). We found most of our cases during that period too. The isolation of the organism from the soil[28] may offer a partial explanation for this seasonality. In our 24 cases it was 6 times more frequent in whites than in the other 2 races, and there were nearly twice as many males as females.

Other reports on the incidence of *M. gypseum* follow: New Orleans, 3 per cent in t. capitis; Indianapolis, 2.6 per cent; San Antonio, 3.5 per cent, Austin, 4 cases of t. capitis in 3 years; Oklahoma City, 3 cases, St. Louis one case and Springfield one case in 3 years; Boston[20] two cases in 6 years. Although it was not reported as occurring in Mexico,[7] we had 3 Latin-Americans in our 24 cases, 2 showing t. corporis and one t. capitis. Valerio[29] reported 9 per cent of 281 cases in Rio de Janeiro. Negroni saw only one case in 5 years in Argentina. In England, Whittle[30] described 2 small epidemics in a Carnation nursery; 8 of 40 employees on one occasion and 13 on another. He was unable to as-

certain the source of the infection however. Walker reported 2 cases in a 3-year survey in England.

Trichophyta

Trichophyton tonsurans infections are still regarded as rarities in this country, as evidenced by the numerous reports of single or several cases in various areas. The organism is most frequently associated primarily with t. capitis. It represented 56.6 per cent of our isolates in t. capitis and 22 per cent of the total tineas. Race was found to be influential in susceptibility to this organism. It was most prevalent in negroes in whom the cultural type was usually identical and different from the types found in the other 2 races. Variety *crateriforme* was the most frequent type in the Latin-Americans, but there were several other distinct varieties found in lesser numbers.

Other reports on the incidence of *T. tonsurans* follow: 2 of 134 cases of t. capitis in New Orleans; 20.3 per cent in San Antonio (Pipkin);[31] 20.6 per cent in Houston (Seale);[31] 12 per cent in Los Angeles;[32] 3 of 32 cases in Phoenix; 2.2 per cent in Indianapolis; 18 cases in 3 years in Michigan;[33] 5 per cent in native born in Manhattan; 89.4 per cent in Mexico; 3.4 per cent in Uruguay; 15.6 per cent in Brazil; 42.8 per cent of all tineas in Puerto Rico[34] and 6 per cent in Jerusalem.

Trichophyton mentagrophytes. This species has been enlarged by Conant *et al.*,[35] to include a number of previous species which are rather similar microscopically but less so as regards colonial appearance. Clinically, moreover, they may involve practically all kinds of dermatophytoses known. Human infection is frequently from an animal source such as livestock, rodents, or laboratory animals. Not only does the fungus produce similar lesions in man and animals, but it has also been cultured from apparently healthy guinea pigs.[36] As is characteristic of infections with zoophilic species, the lesions are frequently inflammatory; kerions being common in t. capitis and Majocchi's granuloma in t. corporis. We have also found *T. verrucosum* and *T. tonsurans* in lesions of the latter type. Our one scalp case was not of an inflammatory nature, although we had a few cases of t. corporis that were. In England where the rural population lives in more intimate contact with livestock, the incidence of these inflammatory lesions is rather high. We have found the organism oc-

casionally in t. cruris where the clinical picture was not distinctive; but the majority of our isolates were from t. pedis, usually of the mild type. Associated with the latter lesions was the downy type of colony in contrast to the granular type found in acute lesions. There was little evidence of "id" eruptions. In the few cases of hand involvement the clinical picture was often that of vesicular dyshidrosis. The use of cycloheximide in Littman's medium greatly facilitated the isolation of the organism from such lesions. It was less frequent in nail infections than *T. rubrum.*

Other reports on the relative occurrence of T. mentagrophytes in tinea capitis follow: 23 of 170 cases in San Antonio;[24] 2 of 134 cases in New Orleans; 9 of 454 cases in Indianapolis; 2 of 288 cases in Boston; 2 of 54 cases in Canada; 2 of 268 cases in Mexico; 15 of 397 cases in Uruguay; 5 of 440 cases in Brazil; 4 of 22 cases in Hawaii; 15 cases in 3 years in England and 50 of 1021 cases in Jerusalem.

The 3 dermatophytes most frequently associated with t. pedis and t. cruris—namely: *T. mentagrophytes. T. rubrum* and *E. floccosum*—are compared in table 7 as regards relative incidence in various parts of the world.

Trichophyton rubrum. This fungus which we found limited to the glabrous skin and nails was by far our commonest isolate in all 3 races. Disseminated cases were not rare, and primary involvement of the buttocks was frequent. It was essentially a disease of adults, especially males. The gross morphology of cultures was extremely variable, but to a certain extent the acuteness of the lesions seemed directly related to the granularity and surface pigmentation of the culture. In Puerto Rico (Carrion)[37] there was no correlation of clinical type with cultural appearance of the organism. The disease in the latter country was also reported readily amenable to treatment in contrast to the chronicity of the disease in the U. S.

Other distributional records follow: 37 cases in 3 years in Austin; 17 cases in St. Louis, 70 cases in Springfield and 50 cases in Oklahoma City in 3 years; 90 cases in 6 years in Manhattan; 33 of 55 cases in the Philippines;[38] 26 of 57 cases in Cuba;[39] 14 of 54 cases in Montreal; 97 cases in 3 years in England; 36 of 47 cases in China[40] and the only isloate in t. cruris in Puerto Rico.[34]

Walker stated that this native fungus of the far East was increasing in England, aided by importation especially from southeast Asia. It was most frequent in industrial areas with equal incidence in both sexes, and there was nail involvement in half the cases. Sisk *et al.*, commented on the long duration of the disease in their areas, it being usually an erythematosquamous but occasionally a vesicular eruption. Significant increases have been noted since World War I in Germany[41] and in Canada.

Epidermophyton floccosum. This organism which attacks the vigorous and well-nourished is widely distributed and seemingly shows no racial preference. Its incidence is also thought to have increased since World War I.[44] However, the occurrence in Galveston was rather infrequent (2.8 per cent) and mostly in t. pedis. It was evidenced by periodical reculturing that some persons infected with *E. floccosum* remained so, the disease persisting in a chronic state with occasional flare-ups. Perhaps there is a similarity to the susceptibility certain individuals shown to tinea versicolor.

Various incidence records follows: 5 cases in 3 years in Austin; 4 cses in Oklahoma City, 4 in St. Louis and 9 in Springfield in 3 years; 5 cases in 6 years in New York City; 3 of 54 cases in Montreal; 22 of 1000 cases in Uruguay; 51 cases in 3 years in; England; and 102 of 326 cases of t. pedis in U. S. army troops in Puerto Rico.[45]

Trichophyton verrucosum. Data are rather recent on the infection with this organism whose culture is now facilitated by the use of enriched media.[46] It is apparently always contracted from livestock, and the lesions are extremely inflammatory. Birt and Wilt[47] have reported on the clinical appearance and histopathology of this infection in Winnipeg. They were able to isolate the organism in 20 of 30 clinical cases. They mentioned that similar suppurative lesions may be produced by *M. canis*, *M. gypseum*, *T. mentagrophytes* and *T tonsuranas.* In a period of 3 years the organism was isolated from 14 cases in a farming area in central Pennsylvania.[48] It was found in 81 of 241 cases of suppurative ringworm in France.[49] Its occurrence in Mexico has also been reported.[50] In England, Rook[57] in a 2-year study reported 105 clinical cases of inflammatory or pustular ringworm exclusive of the feet, with 77 positive cultures of which 40 were *T. verrucosum discoides* and 37 were *T. mentagrophytes.*

Other incidence reports include: St. Louis 1 case, Springfield 3 cases, and Oklahoma City 2 cases in 3 years; Manhattan 1 case in 6 years; England[29] fifty-one cases in 3 years; Brazil 1 case and Uruguay 2 cases.

Trichophyton violaceum. This organism has been reported to be limited to the Semitic race or to populations with large admixtures of Semitic blood.[44] In Texas, 4 cases of t. capitis in adults have been reported by Pipkin.[52] According to Lewis and Hopper[1] it was chiefly found in European immigrants or their siblings in the U. S. Reiss reported 7 cases in a total of 664 tineas at Bellevue Hospital. In Mexico it accounted for 0.8 per cent of 268 cases of t. capitis. In the Philippines there were 2 cases in Chinese boys. In Great Britain, Walker saw 5 cases in 3 years all being imported from India, Egypt or Poland. In Brazil, 48.5 per cent of 442 tineas were *T. violaceum*, all occurring in Italian immigrants. In Uruguay, 17 cases were reported in immigrants from Israel. Sabouraud estimated 15 per cent in Paris. It was reported as the regional type in North Africa[53] and was frequent in North China.[54] *T. violaceum* has been found the most common fungus pathogen in the Ukraine.[55]

The Occurrence of Mixed Infections. It has been our experience that t. capitis cases usually yield a single organism. In one case a Latin-American boy was infected with both *M. canis* and *T. tonsurans*, but after several weeks treatment only the latter was recovered on culture. In another instance, a Latin-American woman and her 6-year-old son had t. capitis due to *T. tonsurans* and t. corporis due to *T. rubrum*. In t. pedis, however, there is frequent association of several organisms apparently growing symbiotically since they appear in the same spots on culture plates. *Candida albicans* and other species of *Candida*, *Geotrichum* and *Rhodotorula* were often found associated with *T. mentagrophytes* or *T. rubrum* in t. pedis and t. cruris.

In the literature, such mention of concomitant infection is rare. Dostrovsky cited 2 cases of t. capitis in which both *T. violaceum* and *T. Schoenleini* were cultured, and another in which both *T. violaceum* and *T. tonsurans* were found. Loewenthal[56] reported a case of combined infection with *Microsporum Audouini* and *M. canis*.

Some distinctive features of this study were:

1. Inclusion of both clinic and private patients; *i.e.*, a consideration of the effect of economic status.
2. Inclusion of substantial numbers of 3 races; *i.e.*, white, negro and Latin-American. This allowed deductions as to race susceptibility.
3. The geographic location plus the latter permitted study of the possible spread of fungus species common to the north of us, and of those prevalent to the south of us.
4. Age and sex were also noted for comparison with other data of this sort.
5. Some attempt was made to interrelate the gross morphology of cultures of the various fungus species with the 3 races and with the clinical condition.

The following assumptions may therefore be made:

1. A true picture of the relative incidence of dermatophyte species of a given area necessitates the consideration of all economic states of the population.
2. The white, negro and Latin-American races show different degrees of susceptibility to infection with certain dermatophyte species. For example: The Latin-Americans do not appear to be susceptible to *M. Audouini*, which may be the reason the species is not spreading southward. The white race shows some susceptibility to infection with *T. tonsurans*, but the present incidence is very low, both in comparison with the relative percentage of other species which parasitize the white race and as regards the incidence of *T. tonsurans* in the other two races.
3. There appears to be no racial barrier to infection with *T. rubrum* whose incidence in our area is apparently higher than in other localities in the U. S. This would seem already to be a significant public health problem, and to indicate the spread of a southern species.
4. There is also no racial antipathy to the zoophilic species *M. canis*, *M. gypseum* and *T. mentagrophytes*. However, the relative incidence of these is not high, and is comparable with that in other areas of the nation.
5. There was noted an association of granular colonies with inflammatory lesions and downy colonies with mild one with *T. mentagrophytes* and *T. rubrum* infections. No

correlation of gross culture morphology with race was observed in *T. rubrum* such as there was in *T. tonsurans.*

6. Our data agree with previous reports that the male is more frequently a victim of dermatophytoses than the female; that t. capitis is essentially a prepubertal disease, the average age being 5–6 with Microspora and slightly higher with *T. tonsurans;* and that there were a few cases of t. capitis in adults due to *T. tonsurans.*

Summary

A study has been made on the superficial dermatomycoses which have been diagnosed at The University of Texas Medical Branch in Galveston during the 5-year period of 1951 through 1955. Statistical data have been assembled regarding species of fungi, sites of infection, sex, race, age, epidemiology, residence, economic status, duration of infection, clinical appearance, and cultural characteristics.

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TABLE 1

Incidence of Superficial Fungi in Galveston

Organism	Total—5 Years		1951	1952	1953	1954	1955
	No.	Per Cent					
Microsporum Audouini	40	3.5	3	0	14	10	13
Microsporum canis	124	10.7	21	20	30	29	24
Microsporum gypseum	24	2.1	3	7	6	5	3
TOTAL MICROSPORA	188	16.3	27	27	50	44	40
Trichophyton tonsurans	253	22.0	67	55	45	35	51
Trichophyton mentagrophytes.	172	14.9	45	29	31	26	41
Trichophyton rubrum	500	43.4	144	94	62	75	125
Trichophyton verrucosum	2	0.2	0	0	0	1	1
Trichophyton violaceum	2	0.2	2	0	0	0	0
Epidermophyton floccosum ...	33	2.8	11	3	4	6	9
Cladosporium Wernecki	3	0.2	0	0	0	1	2
TOTAL OTHERS	965	83.7	269	181	142	144	229
TOTAL	1153		296	208	192	188	269

TABLE 2

Sites of Infection

Organism	Scalp	Feet	Hands	Nails	Buttocks and Groin	T. Corporis (Misc.)	Dissemi-nated	Total Sites
M. Audouini	36	0	1	0	0	11	0	48
M. canis	95	0	1	0	1	27	0	124
M. gypseum	11	0	0	0	0	13	0	24
T. tonsurans	192	0	2	0	3	64	1	262
T. mentagrophytes ..	1	134	5	10	14	13	0	177
T. rubrum	0	122	43	23	176	146	7	517
T. verrucosum	2	0	0	0	0	1	0	3
T. violaceum	2	0	0	0	0	0	0	2
E. floccosum	0	23	0	0	7	9	0	39
C. Wernecki	0	0	2	0	0	1	0	3
TOTAL	339	279	54	33	201	285	8	1199

TABLE 3

Species Incidence in Tinea Capitis

Organism	Total Cases	Cases* Analyzed	Sex		Race			Age Range (Average)	Economic Status		
			M	F	W	N	LA		Cl.	P.P.	Pers.
M. Audouini	36	36	30	6	9	27	0	2–14 (5.9)	29	7	0
M. canis	95	32	21	11	23	3	6	1/12–10† (5.4)	17	15	0
M. gypseum	11	11	7	4	7	3	1	2–12 (5.8)	5	6	0
Total Microspora..	142	79	58	21	39	33	7		51	28	0
T. tonsurans	192	44	29	15	4	21	19	2–13‡ (7.1)	39	5	0
T. mentagrophytes	1	1	0	1	0	0	1	4	1	0	0
T. verrucosum ...	2	2	2	0	2	0	0	6 & 66	1	1	0
T. violaceum	2	2	1	1	0	0	2	11 & 13	2	0	0
Total Trichophyta.	197	49	32	17	6	21	22		43	6	0
TOTAL	339	128	90	38	45	54	29		94	34	0

* Cases analyzed in last 20 months only for *M. canis* and *T. tonsurans*.
† 1 exception—negro female of 47 (L.E.).
‡ 3 exceptions—3 L.A. females of 35, 41, and 62.

TABLE 4

Species Incidence in Tinea Corporis

Organism	Total Cases	Cases* Analyzed	Sex		Race				Age Range	Economic Status		
			M	F	W	N	LA	CH		Cl.	P.P.	Pers.
M. Audouini	12	12‡	9	3	2	10	0	0	2–49	12	0	0
M. canis	29	9‡	1	8	9	0	0	0	5–70	2	5	2
M. gypseum	13	13	8	5	11	0	2	0	1/6–62	5	6	2
Total Microspora..	54	34	18	16	22	10	2	0		19	11	4
T. tonsurans	69	27†	11	16	3	13	10	1	2–68	21	5	1
T. mentagrophytes	171	53	33	20	35	10	8	0	3–70	18	25	10
T. rubrum	500	176	103	73	104	44	28	0	1/6–79	102	59	15
E. floccosum§	33	33	23	10	19	6	5	0	1/2–74	20	8	5
C. Wernecki	3	3	1	2	2	1	0	0	4–38	1	2	0
Total Others	776	292	171	121	163	74	51	1		162	99	31
TOTAL	830	326	189	137	185	84	53	1		181	110	35

* Cases analyzed in last 20 months only—*M. canis, T. tonsurans, T. mentagrophytes, T. rubrum.*
† Eight of these also had t. capitis.
‡ Association of t. capitis not studied.
§ Data as to race unavailable for 3.

TABLE 5

Race and Tinea

Race	No. Patients	Tinea capitis	Tinea corporis	Microsporum* Audouini	Microsporum† canis	Microsporum* gypseum	Trichophyton† tonsurans‡	Trichophyton† mentagrophytes	Trichophyton† rubrum	Trichophyton* verrucosum	Trichophyton* violaceum	Epidermophyton* floccosum	Gladosporium*
White	229	45	185	10	32	18	7	35	104	2	0	19	2
Negro	128	54	84	30	3	3	29	10	44	0	0	6	1
Latin-American	77	29	53	0	6	3	26	9	28	0	2	5	0
TOTAL	434	128	322	40	41	24	62	54	176	2	2	30	3

* 5 years.
† 20 months.
‡ One Chinese also had *T. tonsurans* infection.

TABLE 6

Numerical Incidence of Tineas in Various Localities

Organism	Galveston 1956	New York Reiss 1954	Mexico* Gonzalez Ochoa 1945	Uruguay* Mac-kinnon 1945	Brazil* Rossetti 1941	England Walker 1950	Paris* Sabouraud 1910	Jerusalem† Dos-trovsky 1955
M. Audouini	40	318	0	0	0	1433	149	0
M. canis	124	112	22	319	125	544	12	0
M. gypseum	24	1	0	2	4	8	0	0
Total Microspora	188	431	22	321	129	1985	161	0
T. tonsurans	253	44†	240	15	69	39	186	18
T. mentagrophytes	172	68	2	15	5	174	0	50
T. rubrum	500	90	..	..	..	97	..	..
E. floccosum	33	5	..	..	..	51	..	..
T. verrucosum	2	1	..	17	1	51	..	..
T. violaceum	2	7	2	17	219‡	5	31	830
T. Schoenleini	0	1	2	12	17	42	52	123
Total Others	962	216	246	76	311	459	269	1021
TOTAL	1150	647	268	397	440	2444	430	1021

* T. capitis only.
† Includes 15 Puerto Ricans and an epidemic at a children's home.
‡ Italian immigrants.

TABLE 7

Relative Incidence of 3 Tinea Corporis Organisms

Locality	Authority	Date	Percentage			Total No. Cases
			T. rubrum	T. mentagrophytes	E. floccosum	
Galveston, Texas	Pinkerton, *et al.*	1956	71.0	24.3	4.7	704
Austin, Texas	Polsky	1953	58.7	33.3	8.0	63
St. Louis, Mo.	Sisk, *et al.*	1953	44.7	44.7	10.6	38
Springfield, Mo.	Sisk, *et al.*	1953	61.4	30.6	8.0	114
Oklahoma City, Okla.	Sisk, *et al.*	1953	46.7	49.6	3.7	107
New York, N.Y.	Reiss	1954	55.2	41.7	3.1	163
Montreal, Canada	Danby	1947	33.3	59.6	7.1	42
Uruguay, S.A.	MacKinnon	1950	25.3	50.5	24.2	91
England	Walker	1950	31.4	51.4	17.2	296
Cuba	Armenteros	1953	66.6	5.2	28.2	39
Philippines	Simuangco	1955	43.3	55.6	1.1	90
India	Chakraborty	1955	63.0	2.36	33.23	700*
V.A., Los Angeles, Cal.	Burke, *et al.*†	1949	32.2	65.6	2.2	180
U.S. Army in Puerto Rico	Campbell, *et al.*†	1955	39.0	30.0	31.0	326
Literature	Hopkins, *et al.*†	1947	36.0	47.0	17.0	1700

* *T. violaceum* accounted for 1.41 per cent.
† Feet only.

THE EFFECT OF SOME SELECTED ANTIBIOTICS ON EXPERIMENTAL CANDIDIASIS*

MARY E. PINKERTON AND MARCEL PATTERSON

The etiology of many of the gastrointestinal symptoms that are noted after antibiotic therapy is unknown. This is particularly true of pruritus ani and the sore tongue and mouth that occasionally occur. Among the numerous theories that have been offered is that related to an apparent numerical increase of *Candida albicans* in the gastrointestinal tract after such treatment. We have been impressed by the lack of these annoying symptoms following the use of oral neomycin.[1] Therefore, it seemed of interest to investigate the effects of neomycin on experimental candidiasis in mice following the technic of Seligmann (1952). In addition several other antibiotics were studied for comparison.

Materials and Methods

Organism. A highly virulent strain of *Candida albicans* isolated from the gastric washings of a patient suffering from generalized candidiasis and tuberculosis was used. A loopful of this stock culture on Sabouraud's agar was inoculated into a tube of Sabouraud's broth and incubated at 37° C. for 24 hours. One-tenth ml. of the latter was used as inoculum per tube of Sabouraud's agar which was incubated for 72 hours at 37° C. The resultant growth was scraped off into normal saline, centrifuged at 1500 rpm. for 20 minutes and 0.1 ml. of packed cells was used per 2.4 ml. of saline or saline-antibiotic mixture. The virulence of the culture gradually decreased after a number of agar transfers.

Antibiotics. The following antimicrobial agents were utilized: neomycin sulfate[2], chlortetracycline hydrochloride[3], tetracycline hydrochloride[4], nystatin[5], filipin[6], and endomycin[7]. Stock mixtures were made with normal saline

* From the Departments of Dermatology and Internal Medicine, The University of Texas Medical Branch, Galveston, Texas.

[1] The trade name of The Upjohn Company for neomycin is Mycifradin sulfate.

[2] Prepared and supplied by the Research Laboratories of The Upjohn Company, Kalamazoo, Michigan.

[3] The trade name of Lederle Laboratories Division, American Cyanamid Company, for chlortetracycline is Aureomycin.

[4] The trade name of Lederle Laboratories Division, American Cyanamid Company, for tetracycline is Achromycin.

[5] The trade name of E. R. Squibb & Sons, Olin Mathieson Chemical Corporation, for nystatin is Mycostatin. (Lot No. St. 695–714/15–1–B.)

[6] Prepared and supplied by the Research Laboratories of The Upjohn Company, Kalamazoo, Michigan. Isolated by Dr. David Gottlieb from *Streptomyces filipinensis.*

[7] Prepared and supplied by the Research Laboratories of The Upjohn Company, Kalamazoo, Michigan. Isolated by Dr. David Gottlieb from *Streptomyces enders.*

and diluted so that the desired dose was present in 0.2 ml., the dose per mouse. Immediate inoculation followed the combination of the drug mixture and packed *Candida* cells.

Host. Miscellaneous white mice of CFW and CFl strains, both sexes, age 1 to 4 months, and weighing 25 to 35 gms., were used in these experiments. There was no obvious variation in susceptibility to *C. albicans* due to strain, sex, age or weight of the mice. With a few exceptions to be mentioned later, a single intraperitoneal injection was given. A control group receiving only the pathogen was used with each experiment. Limited drug controls were also employed. The mice were checked daily for 30 days, and autopsies were performed on all of those dying within that period and on a few of the survivors. Histopathological sections were made of vital organs of representative mice of the various test groups.

Results

The experimental data are summarized in Tables 1 and 2.

Fatality.

Pathogen controls. Thirty-two (84.2 per cent) of the 38 pathogen controls died. Twenty-two (68.7 per cent) of these deaths occurred within 48 hours after inoculation with *C. albicans*. All animals appeared ill shortly after injection, manifesting chills and lethargy.

Neomycin. At a level of 4 mgms. per mouse no deaths occurred. But with the drug-pathogen mixture 4 (80 per cent) of 5 mice died within 48 hours, whereas only 2 (40 per cent) of the 5 pathogen controls died in that period and 3 (60 per cent) within 30 days. At 2 mgms. per mouse of neomycin plus pathogen, 7 (77.8 per cent) of 9 mice died within 72 hours as did 8 (80 per cent) of 10 pathogen controls. There were no further deaths in the former group whereas all of the latter group were dead by the 16th day. At 1 mgm. per mouse of neomycin plus pathogen, 4 (28.5 per cent) of 14 mice died within 72 hours compared to 9 (64.2 per cent) of 14 pathogen controls. Eventually 11 (78.5 per cent) of 14 mice died in both groups, but in the pathogen control group 81.8 per cent of the deaths occurred within 48 hours, while the antibiotic-pathogen group's deaths occurred gradually over a period of 12 days.

Two of 3 mice receiving 0.5 mgms. of neomycin plus pathogen died slightly more rapidly than did the same number of pathogen

controls. Of 3 infected mice receiving neomycin (1 mgm./mouse) on 2 successive days, 2 died on the 10th and 11th days. Only one of the 3 pathogen controls died and that occurred on the 10th day.

Tetracyclines. Chlortetracycline and tetracycline at dosage levels of 2 mgms. per mouse caused no deaths, but when combined with *C. albicans* they were fatal to all the mice as was also the pathogen alone. In the case of chloreteracycline 7 (87.5 per cent) of 8 deaths occurred in the first 72 hours compared to 8 (80 per cent) of 10 deaths in the controls. With tetracycline, the 72 hour death rate was identical in the two groups, 8 (80 per cent) of 10 mice. There was no significant difference in mortality with pathogen plus chlortetracycline at 1 mgm. per mouse and with the pathogen alone. All but one of the animals died in each group.

In one experiment using 0.5 mgm. per mouse of chlortetracycline plus pathogen, there was 1 death in this group on the 26th day and no deaths in the 3 pathogen controls. In 4 mice of another group given 1 injection of pathogen and 0.5 mgms. of

TABLE 1

Influence of Antibiotics on Fatality of Experimental Candidiasis in Mice

Test Organism	Antibiotic	Dose mgms.	No. of Exps.	No. Mice		Percent Fatalities
				Inoc.	Dead	
C. albicans	.....	..	7	38	32	84.2
C. albicans	Neomycin	4	1	5	4	80.0
C. albicans	Neomycin	2	1	9	7	77.8
C. albicans	Neomycin	1	3	14	11	78.6
C. albicans	Neomycin	0.5	1	3	2	66.6
.....	Neomycin	4	1	2	0	0.0
.....	Neomycin	2	1	2	0	0.0
C. albicans	Aureomycin	2	1	8	8	100.0
C. albicans	Aureomycin	1	2	10	9	90.0
.....	Aureomycin	2	1	2	0	0.0
C. albicans	Achromycin	2	1	10	10	100.0
.....	Achromycin	2	1	2	0	0.0
C. albicans	Mycostatin	2	2	17	13	76.5
C. albicans	Mycostatin	1	1	5	3	60.0
C. albicans	Mycostatin	0.5	3	10	0	0.0
.....	Mycostatin	2	1	2	2	100.0
C. albicans	Filipin	0.5	1	4	2	50.0
C. albicans	Endomycin	0.5	1	4	4	100.0

* Standard dose of *C. albicans* approximately 320 million cells. Antibiotic dose in mgms. per mouse.

drug daily for three days (total dose 1.5 mgms.) there were 3 deaths. These occurred on the 11th, 14th and 19th days.

Nystatin. At 2 mgms. per mouse, nystatin alone caused death in both of 2 mice. When combined with pathogen there were 13 (70.5 per cent) deaths in 17 mice within 72 hours as compared to 11 (57 per cent) deaths in the 19 pathogen controls. Thereafter, there were no deaths in the 4 remaining mice of the antibiotic-pathogen group while in the latter pathogen control group all but one eventually died. At 1 mgm. per mouse, the mortality rate was the same between the 2 groups the first 72 hours (3 of 5 mice died) with 1 more death occurring in the pathogen control group on the 8th day and no further deaths in the antibiotic-pathogen group.

At 0.5 mgms. per mouse there were no deaths in 10 mice given antibiotic plus pathogen, and there were no clinical toxic symp-

TABLE 2

Influence of Antibiotics on the Fatality Rate of Mice Injected with Candida albicans

Pathogen	Antibiotic	Dose mgms.	No. Inj.	No. Dead	Day of Death 1	2	3	4	5	6	7	8	9	10	11-26
C. alb.*	.....	.	38	32	11	11	2	0	1	2	2	0	0	0	1
C. alb.	Neomycin	4	5	4	3	1	0	0	0	0	0	0	0	0	0
C. alb.	.....	.	5	3	1	1	0	0	0	1	0	0	0	0	0
C. alb.	Neomycin	2	9	7	3	3	1	0	0	0	0	0	0	0	0
C. alb.	.....	.	10	10	3	4	1	0	1	0	0	0	0	0	1
C. alb.	Neomycin	1	14	11	1	2	1	1	1	0	0	2	0	1	2
C. alb.	.....	.	14	11	4	5	0	0	0	1	0	1	0	0	0
C. alb.	Aureomycin	2	8	8	0	3	4	0	1	0	0	0	0	0	0
C. alb.	.....	.	10	10	3	4	1	0	1	0	0	0	0	0	1
C. alb.	Aureomycin	1	10	9	2	3	1	1	0	0	0	1	0	0	1
C. alb.	.....	.	9	8	3	4	0	0	0	0	0	1	0	0	0
C. alb.	Aureomycin	0.5[1]	4	1	0	0	0	0	0	0	0	0	0	0	1
C. alb.	Aureomycin	0.5[3]	4	3	0	0	0	0	0	0	0	0	0	0	3
C. alb.	.....	.	3	0	0	0	0	0	0	0	0	0	0	0	0
C. alb.	Achromycin	2	10	10	2	6	0	0	0	0	0	2	0	0	0
C. alb.	.....	.	10	10	3	4	1	0	1	0	0	0	0	0	1
C. alb.	Mycostatin	2	17	13	10	2	1	0	0	0	0	0	0	0	0
C. alb.	.....	.	19	18	3	6	2	0	3	0	0	0	1	1	2
C. alb.	Mycostatin	1	5	3	1	1	1	0	0	0	0	0	0	0	0
C. alb.	.....	.	5	4	1	2	0	0	0	0	0	1	0	0	0
C. alb.	Mycostatin	0.5	10	0	0	0	0	0	0	0	0	0	0	0	0
C. alb.	.....	.	10	6	2	2	0	0	0	0	1	0	0	0	1
C. alb.	Filipin	0.5	4	2	0	2	0	0	0	0	0	0	0	0	0
C. alb.	Endomycin	0.5	4	4	0	3	0	1	0	0	0	0	0	0	0
C. alb.	.....	.	3	0	0	0	0	0	0	0	0	0	0	0	0

* Standard dose of *C. albicans* approximately 320 million cells. Antibiotic dose in mgms. per mouse. Raised figures after Aureomycin 0.5 indicate 1 and 3 doses respectively.

toms. Four (40 per cent) of 10 pathogen control mice died within 48 hours and eventually two others on the 7th and 16th day.

Other antibiotics. Filipin and endomycin in 0.5 mgms. per mouse doses plus pathogen killed 50 per cent and 100 per cent (4 mice in each group) respectively. In this experiment there were no deaths among the 3 pathogen controls. The animals receiving endomycin-*Candida* mixtures were acutely ill until their deaths.

Pathology

Pathogen controls. Those mice dying within 48 hours showed mild inflammation of the peritoneal wall and a few tiny white *Candida* colonies visible in the liver, kidneys and pericardium but no involvement of the spleen. Microscopically there were granulomas in the omentum with budding and mycelial forms of the organism. A moderate number of colonies of both types of growth was noted in the cortex and medulla of the kidneys and in the myocardium suggesting blood borne metastases. A very few organisms were seen in the lumen of the gut.

Several controls living beyond 30 days were sacrificed. All showed peritoneal adhesions, presumably the results of healed infections with *C. albicans*. One mouse killed 80 days after infection showed adhesions of the liver and spleen to the stomach and peritoneal walls. Retroculture of *C. albicans* was successful.

C. albicans plus neomycin at 2 mgms. per mouse. There was minimal inflammation of the peritoneal walls, although a few *Candida* colonies were found in the peritoneal walls, in the pericardium and in the kidneys. There were no colonies in the liver or spleen. Microscopically the lesions were similar to those seen in mice receiving pathogen alone. One mouse sacrificed 116 days after receiving 2 mgms. of neomycin plus pathogen showed no lesions and retroculture of *C. albicans* was unsuccessful.

C. albicans plus chlortetracycline at 2 mgms. per mouse. In 48 hours there were numerous *Candida* colonies on the surfaces of the liver, omentum, kidneys, intestines, peritoneal wall and pericardium. There was also a tendency toward fusion of adjacent organs by direct extension of the growth of *C. albicans;* such as liver to stomach and diaphragm, and spleen to stomach. This tendency was intensified in later deaths. Microscopically, there was peri-renal involvement suggesting that the *Candida* had infiltrated from the exterior of the kidney rather than being blood

borne. There was also involvement of the wall, mucosa and muscularis of the large intestine. In addition, there were definite granulomatous lesions of the liver and peritoneal walls. Similar results were noted in the tetracycline-pathogen group of animals.

C. albicans plus nystatin at 2 mgms. per mouse. Mice dying within 24 hours occasionally showed a few tiny *Candida* colonies in the liver and the kidneys and slight inflammation of the peritoneal wall. The one mouse succumbing at 72 hours showed a slight subcutaneous abscess at the point of inoculation, but there were no obvious internal lesions. Mice surviving 30 days after inoculation were sacrificed and found to show no signs of infection.

C. albicans plus filipin and endomycin. Mice succumbing after treatment with filipin showed mild inflammation and a few *Candida* colonies. Two mice sacrificed after 30 days showed intraperitoneal adhesions as did the pathogen controls. The victims of endomycin therapy showed intense inflammation of mucosal surfaces but no *Candida* colonies.

In summary: the pathological picture with *Candida* alone and with Candida plus neomycin is similar and different from that produced by either of the two tetracyclines plus pathogen. In the latter the growth of *C. albicans* was more abundant with direct invasion of tissues, whereas in the former two groups there was a tendency to a few discrete colonies with metastases via the blood rather than by direct spread. These data from this limited study suggest to us that damage to the mucosa by the tetracyclines allows for the enhanced growth of *C. albicans* that was noted.

Discussion

The various theories as to the mechanism by which certain antibiotics potentiate the growth of *C. albicans in vivo* include:

1) *Increase of Candida organisms due to lack of competition*, since most of the normal bacterial flora in the intestinal tract is attacked by antibiotics (Robinson, 1954).

2) *Direct stimulation of the growth of C. albicans.* Results of *in vitro* tests are not in agreement (Moore, 1951 and Minieri *et al.*, 1953). *In vivo* tests in the peritoneal cavity do seem to indicate that there is increased infection following the tetracyclines-*Candida* inoculations. It is difficult to determine if the end

result is achieved through potentiation of the organism, or through decreased resistance of the tissues. Our results we feel suggest the latter, since mortality rates were very similar in all groups (excluding nystatin) yet the pathological picture was one of greater growth and invasion of *C. albicans* in the tetracyclines groups.

3) *Vitamin B-complex deficiency*. Leitner (1950) and Harris (1950) have claimed success in prevention and treatment of untoward symptoms by the administration of Vitamin B-complex concurrently with anibiotics. On the other hand Sulzberger and Baer (1955) were of the opinion that there was no adequate evidence of the prevention or elimination of such complications by the administration of B-complex vitamins. Our personal experience has led us to a similar conclusion.

4) *Tissue alterations attributable to the tetracyclines*. Kligman (1952) felt that the tetracyclines caused mucous membrane irritation, and regarded the influence of *C. albicans* as negligible. Winter and Foley (1956) speculated on the altered tissue response occasioned by the tetracyclines and concluded that the enhancement of the pathogenicity of *C. albicans* was associated with "decreased hematogenous dissemination," They were impressed by the direct invasion of the kidney capsule, but found that other organs were essentially unaffected in their experiments with a relatively avirulent strain of *C. albicans*.

We would like to suggest that the so-called "hairy tongue" may be due to a direct stimulating effect on papillae growth by the antibiotic secreted in the saliva.

In experimental candidasis one is amazed by the relative rapidity of death in a large percentage of cases. Intravenous inoculation of *C. albicans* in mice produced death in 24 hours; but similar experiments with *C. parakrusei* produced innocuous effects (Shapiro *et al.*, 1956). These observations suggest a toxic factor. Salvin (1952) found that a number of species of *Candida* and *Saccharomyces cerevesiae* produced endotoxins, but he was unable to correlate toxin-producing ability and pathogenicity.

Our experiments were designed toward detecting a lessening of virulence of a pathogenic strain of *C. albicans* by neomycin, whereas Seligmann (1952) proved the intensification of virulence of a relatively non-pathogenic strain with tetracyclines. Duke and Tettenbaum (1955) maintained a constant drug dos-

age and varied the size of the inoculum, in contrast to our method of using a fixed inoculum and varying the drug dosage. They noted a quantitative relationship in regard to the intensification of *C. albicans* infection by tetracyclines. We found a similar situation regarding the elimination of pathogenicity of our strain of *C. albicans* by nystatin.

Spaulding *et al.*, (1956) have been fairly successful in creating complete intestinal antisepsis with a combination of neomycin and nystatin, and have found that the addition of polymyxin B accentuates the effect.

The significance of *Candida* infections in the gastrointestinal symptomatology that occasionally occurs cannot be ascertained at this time. One might postulate on the basis of our experiments that neomycin produces less symptoms because there is less direct irritating effect on mucosal surfaces. But our results do not explain why chlortetracycline is much more likely to produce symptoms than tetracycline, since there was no significant difference between the two drugs in these experiments.

Systemic infections by *Candida* in humans under treatment with the tetracyclines are rare, and we have noted no striking improvement in gastrointestinal symptoms when treatment is directed toward elimination of *C. albicans*. Perhaps Kligman (1952) is correct in assuming that the influence of *C. albicans* in the development of this symptomatology is usually negligible.

Summary and Conclusions

Neomycin, chlortetracycline and tetracycline were found to be innocuous intraperitoneally at a level of 2 mgms./mouse. On combination with *C. albicans* they produced rapid illness in all of the mice, as did the pathogen alone. Nystatin at the same level was toxic, but upon combination with the pathogen it protected some of the mice. At a level of 0.5 mgms./mouse plus *Candida* it was nontoxic and protected all of the mice.

Although the tetracyclines combined with the pathogen produced the same toxic symptoms and death mostly within 48 hours as did also the pathogen controls, the former produced earlier lesions which were superficial and due to direct penetration. This feature was not manifest by neomycin. It is suggested that direct tissue damage is a potent aspect of the sequelae after therapy with the tetracyclines.

The combination of neomycin and *C. albicans* gave essentially the same results on intraperitoneal injection as did the pathogen alone so far as the total fatality was concerned. The only suggestion of a possibily beneficial effect was that at the 1 mgm./mouse level the average time of death was delayed.

The single tests with endomycin and filipin indicated the relative toxicity of these drugs making them unsuitable for testing by this method.

Acknowledgments

The authors wish to express their thanks to Dr. Edith D. Box for furnishing laboratory facilities and mice for this investigation. We are also grateful to Mrs. Betty S. Head for histological preparations and to Dr. Vern E. Stembridge for interpretation of the slides. Mice were also secured with funds from the Department of Internal Medicine. Antibiotics were furnished by the various biological houses manufacturing them.

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PROCEEDINGS OF THE TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Abstracts

Of Some of the Papers Read at the Fall Meeting of the Texas Branch, Society of American Bacteriologists

November 23–24, 1956

The University of Texas
Austin, Texas

Bacteriophage Activity In Pseudomonas. Major Jose A. Rivera and Russell E. Brame, Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas.

Studies on *Pseudomonas* and their pigments showed the presence of bacteriophage activity. The relation of this activity to the production of pigments was studied both *in vivo* and *in vitro*.

Our study included lysogenicity and the effect of inducing phage production by ultraviolet light. Irradiation effect was temporary in certain cells, but permanent inhibition occurred in others. Lysogenic colonies survived better after ultraviolet irradiation and more pigment concentrated around these colonies. In some instances, ultraviolet irradiation induced strains to better phage production as indicated by an increase in the number of plaques and a decrease in the number of colonies. Variations in character, appearance, size, and shape of the plaques are entities in differentiating one phage from another. These entities will serve to classify different strains of *Pseudomonas* according to bacteriophage activity.

It was observed that as lysogenicity increased, pigment production decreased and that phage activity in *Pseudomonas aeruginosa* occurs frequently. Our statistics show a high incidence of phage activity, totaling 61.8 per cent of all strains isolated from cultures of infections in burned patients during the last six months.

Direct Photometry of Diffusing Precipitin Systems for Characterizing Proteins. William G. Glenn, Department of

Microbiology, School of Aviation Medicine, USAF, Randolph Air Force Base, Texas.

Accurate rapid photometric quantitation of agar columns with diffusing precipitin systems has been made possible by the Serum Agar Measuring Integrator (SAMI) developed in our laboratory.

The kinds of data that can be obtained for chracterizing proteins are: (1) diffusion rate, (2) zonal density, (3) precipitin zonal width, (4) slope of the zonal turbidimetric precipitin curve from trailing edge to equivalence.

Bacteriophage Sensitivity in Staphylococci. Eugene D. Rosenblum and Warren Jackson, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

One hundred and forty-three strains of staphylococci, freshly isolated from hospital patients, were tested with a series of staphylococcus typing bacteriophages, and classified as bacteriophage reactive and nonreactive. Bacteriophage reactive strains included those typeable with routine test dilutions as well as those lysed or "inhibited" (Williams and Rippon, 1953) by full strength bacteriophage suspensions. The coagulase negative staphylococci (61 strains) were all bacteriophage nonreactive. Among the coagulase positive strains, 66 (91.7 per cent) of the 72 pigmented strains were bacteriophage reactive, but only two of the ten white coagulase positive strains reacted to bacteriophage. It would seem, therefore, that a high degree of correlation exists between pigmentation, coagulase production, sensitivity to bacteriophage, and by inference, to virulence.

This correlation seems to apply only to freshly isolated staphylococci, and is much less pronounced in laboratory stock cultures or in the above strains when retested after several months storage. The strains maintained on artificial media tend to lose properties such as pigmentation and coagulase production more rapidly than their sensitivity to bacteriophage.

A Simple Quantitative Determination of Anaphylactic Shock In Mice. Lt. William E. Harris and Major John D. Fulton, Department of Microbiology, School of Aviation Medicine, USAF, Randolph Air Force Base, Texas.

A marked rise in hematocrit of anaphylactic mice has been

observed. By utilizing a modification of the standard microhematocrit technique, it was possible to develop a quantitative procedure for estimating the severity of anaphylaxis in mice. It was shown that the degree of hematocrit rise is a linear function of the $\log_{10}$ dose challenge antigen concentration over a given dose range. This procedure is recommended for drawing comparisons in quantitative studies of generalized hypersensitivity in experimental animals.

Bacteriophage Clearance in Zymosan-Treated Animals. S. Edward Sulkin, Eugene D. Rosenblum and Richard A. Finkelstein, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

In confirmation of previous reports, inactivation of Staphylococcus phage (Type 53, Blair) has been demonstrated in fresh normal rat and rabbit serum. This inactivation could be prevented by pre-treating the serum at 17° C, with zymosan, a yeast cell wall preparation, which has been reported to adsorb properdin. An evaluation of the role of the properdin system in the *in vivo* inactivation of bacteriophage was attempted by determining the amount of circulating bacteriophage in zymosan-treated and control rabbits at intervals following intravenous administration of approximately 10^9 plaque-forming particles. A rapid removal of circulating phage could be demonstrated within 2.5 minutes in both groups of animals. However, in animals pre-treated with zymosan, plaque counts were consistently four to more than tenfold higher than in control animals. Preliminary observations indicate that serum from rabbits treated four hours previously with zymosan is less virucidal and bactericidal (against *Shigella dysenteriae*) than normal rabbit serum.

The results of this study constitute presumptive evidence that the properdin system may be involved in the *in vivo* inactivation of bacteriophage, but additional confirmation is desirable.

The Lethal Effects of Gamma Radiation on *Neurospora* Conidia. Captain Robert D. Gafford, Department of Microbiology, School of Aviation Medicine, USAF, Randolph Air Force Base, Texas.

Multinucleate and uninucleate conidia of *Neurospora crassa* were exposed to varying doses of 1.1 Mev gamma radiation from

a 1200 curie Co^{60} source. Spores were exposed in aqueous suspension and in the lyophilized state. Consideration of the survival curves support the following conclusions. Two processes each with a characteristic target number and inactivation constant are effective in killing spores irradiated in aqueous suspension. The first process can best be explained on the basis of a "diffusion model," the second on the basis of a "direct hit model." Only the second of these two processes is effective in killing spores irradiated in the lyophilized state.

Factors Influencing Colonial Morphology of Coagulase Positive Staphylococci in a Semi-liquid Medium. Richard A. Finkelstein and S. Edward Sulkin, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

A procedure has been devised by which coagulase positive and coagulase negative staphylococci can be distinguished culturally. In a soft, 0.15 per cent, agar medium, colonies of staphylococci are feathery, somewhat diffuse, and elongate. When normal or immune plasma or serum is included in the medium, coagulase positive strains produce compact spherical colonies. The colonies of coagulase negative strains are not similarly modified. Forty-four strains of coagulase positive and negative staphylococci have been examined by this procedure with complete correlation between their coagulase activity and morphology in serum soft agar. The colony compacting activity of normal serum appears to be related to its natural antibody content. It is stable to moderate heat, separates with the globulin fraction, and can be removed by adsorption with coagulase positive strains. In addition, the colony compacting titer rises on immunization. The ability of immune sera to cause compact growth of coagulase positive colonies may be a more sensitive indication of their antibody content than standard agglutination procedures.

This method may be useful in analyses of coagulase and/or antigenic variation of staphylococci, and may, with suitable modification, be useful in studies of antigenic variation in a variety of other bacteria.

Some Microbial and Chemical Characteristics of Blackland Soils. Ralph J. Hervey, Texas Agricultural Experiment Station, Temple, Texas.

In studies on the microbial ecology of typical Texas Blackland soils (highly calcareous, heavy clays), the major groups of soil microorganisms were enumerated and various inherent chemical components, and physical conditions determined. Soil samples from various depths were collected from widely separated sites under continuous cotton, grass or virgin conditions.

Relative to total microbial population numbers reported for other soils throughout the U. S. total microbial populations (bacteria and actinomycetes) for many soils in this area are high. Fungus populations, however, are relatively low.

Blackland soils from widely separated sites, at different profile depths and under different crops and "seasonal and weather periods" of the year, vary considerably in pH, carbonates, organic matter, total N, nitrates and size of microbial populations. The number of fungi, actinomycetes and bacteria in Houston Black Clay is generally greatest during the "warm-wet" period (February through June) and smallest during the "hot-dry" period (July through September). The average number of "total microbes" during the "warm-wet" period at 0-6 and 6-12 inches was 49 and 31 million per gram; during the "hot-dry" period counts avaraged 18 and 9 million per gram. Fungus populations averaged 110,000 per gram. H.B.C. generally harbors larger microbial populations, and higher contents of organic matter and total N than Austin clay.

Differentiation of Streptococci in Mixed Bacterial Populations by Use of Fluorescent Antibody. Sidney Halperen, Paul Donaldson, and S. Edward Sulkin, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

Preliminary experiments were designed to adapt the Coons technique to rapid clinical laboratory identification of beta hemolytic streptococci in upper respiratory tract secretions and other specimens. Each of the artificially contaminating organisms was mixed with type 18, group A streptococcus and stained with fluorescent antibody. The organisms used for these mixtures included *Klebsiella pneumoniae*, *Neisseria sicca*, *Micrococcus pyogenes*, *Diplococcus pneumoniae*, and *Hemophilus pertussis*, as well as streptococci of groups B, C, D, and G. A rabbit anti-type 18, group A streptococcus serum and a Lederle anti-group A serum were

used. Examination of the same fields under ultraviolet illumination and dark field showed staining of the organisms for which antisera were specific and cross reactions only with group C streptococci. Five other types of group A streptococci gave similar intensities of specific fluorescent staining. Interfacial precipitin tests during the process of fluorescein conjugation demonstrated an insignificant twofold loss in titer. Paper electrophoresis gave evidence that some albumin and beta globulin remained with the gamma globulin after ammonium sulfate precipitation, but only gamma globulin was in the conjugate.

Description and Some Uses of the Nossal Cell Disintegrator. Robert P. Williams, Davis Hawkins, Jr., and Walter W. Taylor, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

The disintegrator described was constructed according to the plans of Dr. P. M. Nossal (*Austral. Jour. Biol. and Med.* 31: 583, 1953). The machine operates on the principle of an ultra-rapid shaker with a shaking arm oscillating at 12,000 times per minute. In practice we have placed in the stainless steel shaking capsule, 1–2 gms. of wet bacterial cells, 10 gms. of "Superbrite" glass beads, and enough liquid to fill the capsule about three-fourths full. The principal limitation is temperature rise. Shaking for 60 seconds results in a temperature rise of 25° C. If the capsule and its contents are cooled to 0° C., then shaken for 15 seconds, the temperature rises only 8° C. To obtain longer periods of disintegration, we shake the suspension for 15-second intervals followed by cooling to 0° C. Ninety per cent of *Bacillus cereus* spores are destroyed in 120 seconds, and the amount of nitrogen present in the supernatant after centrifugation at 3,500 x g for 30 minutes, increases 10 to 15 times. By use of the apparatus, we have prepared cell free *Serratia* pigments, various dehydrogenase enzymes, and disrupted bacterial cells for easier deoxyribonucleic acid extraction.

Cultivation of Human Epidermoid Carcinoma Cells (strain HeLa) in Citrated Pooled Human Plasma. Craig Wallis, Rae Allen, and S. Edward Sulkin, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

Human epidermoid carcinoma cells (strain HeLa) have been cultivated in citrated human plasma with resultant characteristic growth consisting of discrete, spindle-like cells with nuclei and cytoplasm free of granules. These cells disperse readily after short (15 minutes) exposure to trypsin and appear in the counting chamber as isolated, easily enumerated cells. In contrast, HeLa cells grown in the presence of human serum show a colonial type of growth which merges into a confluent sheath. In addition, the cytoplasm and nucleus of the individual cells present a granular appearance. After prolonged trypsinization serum-grown cells do not separate readily and the presence of clumps in the cell suspensions makes accurate counting difficult. HeLa cells propagated in pooled human plasma promptly revert to serum type cells when human serum is substituted for plasma.

The availability of outdated plasma in areas where blood banks are in operation makes the cultivation of HeLa cells in citrated human plasma a convenient and economical procedure for use in diagnostic and research work with viral agents.

Rapid Indentification of *Monilia* (Candida) *albicans*. W. J. Fahlberg, C. D. Dukes and R. K. Guthrie, Department of Microbiology, Baylor University College of Medicine, Houston and Department of Biology, North Texas State College, Denton.

Monilia albicans when grown on human blood agar under 8–10 per cent CO_2 tension at 37° C., gives rise to filamentous-like colonies within 48 hours. In a study of 149 strains isolated in the laboratory and identified as follows, *C. albicans* (87 strains), *C. tropicalis* (38 strains), C. *krusei* (9 strains), *C. stellatoidea* (12 strains) and *C. pseudotropicalis* (3 strains), the false-positive and false-negative errors totaled 7.4 per cent. A comparison with Nickerson's chlamydospore agar, Nickerson's "Ortho" medium and Litman's oxgall agar indicated the blood agar—CO_2 method to be significantly more accurate. Serological tests employing chlamydospore and yeast cell antisera prepared in the rabbit failed to yield specific agglutination.

Amino Acid Metabolism of *Serratia marcescens*. James A. Green and Robert P. Williams, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

The dynamic biosynthesis of certain amino acids related to the tricarboxylic acid was investigated. Carbon-14 labeled CO_2 was used as an indicator of biosynthesis. The organisms were grown in minimal medium in a closed, aerobic atmosphere. At certain time intervals aliquots were removed for quantitative and isotopic determinations of the amino acids. The results indicated that CO_2 was incorporated extensively into the bacteria, the major portion appearing in phospholipids, peptides, nucleic acids, and protein components. The amino acids labeled were those associated with the tricarboxylic acid cycle through aspartic and glutamic acids. With one exception the pattern followed the generally accepted one for amino acid biosynthesis. As the exception valine was labeled to the same extent as isoleucine suggesting that these two amino acids might have a common precursor in this organism. Chromatography followed by radioautography of isolated organic acids demonstrated that *S. marcescens* possesses a functioning tricarboxylic acid cycle. Citric, fumaric, malic, and succinic acids were isolated and found to be labeled. As the incubation time increased so did the extent of labeling in the amino acids. This fact demonstrated that carbon dioxide was directly involved in the biosynthesis of certain amino acids.

Improving the Efficiency of Screening for Antibiotic Filtrates. Chao-Han Pan and J. W. Foster, Department of Bacteriology, The University of Texas, Austin, Texas.

A combination of very small seed inoculum and a very thin single layer (0.7 mm.) of seeded agar improved appreciably the ability of the agar-diffusion method to detect inhibitory concentrations of antibiotics. With known antibiotics applied to filter paper discs, and using *Micrococcus pyogenes* var. *aureus* as a test bacterium, it was possible to detect ¼ to ⅛ the concentration of the antibiotics that were detectable by conventional screening methods. The concentration of inoculum was that which just allowed discreter colony formation in the agar after incubation. The above procedure and the conventional double-layer-heavy inoculum procedure were compared during screening of 142 actinomycetal filtrates. Using *M. pyogenes* as the indicator organism, the conventional double-layer procedure detected only 42 positive inhibitory filtrates; the modified procedure 69, an increase of 64 per cent. With *Aerobacter aerogenes* as test bacterium the

figures were 2 and 9 positive respectively, an increase of 450 per cent. This sensitive method makes it possible to detect organisms producing lower concentrations of antibiotics than can ordinarily be detected and which ordinarily would be discarded.

Titrations of C'4 and C'3 Components of Complement in Immune Guinea Pigs. Don Hilton, University of Texas Medical Branch, Galveston, Texas, and R. K. Guthrie, North Texas State College, Denton, Texas.

Total complement, C'3 and C'4, were titrated in blood serum from young, uninjected guinea pigs. The guinea pigs were then injected with egg albumin, in a series of three injections within one week. Total complement, C'3 and C'4, were again titrated in the blood serum from these animals taken at one and two weeks after the last injection. Titers of the titrations were compared with titers from the same animals before injection. Uninjected animals were bled at the same time intervals and titrations performed. Comparisons of the titration results showed that in animals receiving injections of egg albumin, the titers of C'3 and of C'4 were considerably elevated after a two week interval, while the titers of these components remaind approximately stationary. Titers of these complement components in infected animals showed a rise in the titers of C'3 and C'4 in these animals comparable to the rise shown in the injected animals.

Coagulase Production. Judith Marston and W. J. Fahlberg, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

The production of coagulase by strains of *Staphylococcus aureus* has been readily accomplished in brain heart infusion broth, with mineral supplement though high titers of activity were not always maintained.

Investigation of factors which might be important in obtaining and maintaining high coagulase activity showed that (1) addition of 5 per cent lactose or 5 per cent sorbitol to the basal medium increased the titer; sorbitol was more effective, (2) increase in pH to the acid side during carbohydrate breakdown seemed to be responsible for a drop in coagulase activity, and (3) shaking of the cultures during incubation resulted in a loss of all activity.

Studies on the Nutrition of the Methane Utilizing Bacterium, *Pseudomonas Methanica*, nov. comb. E. R. Leadbetter and J. W. Foster, Department of Bacteriology, The University of Texas, Austin, Texas.

In a salts-methane medium in 50 per cent methane-50 per cent air, this organism grows poorly unless a cold water extract of agar is added (Dworkin and Foster, *J. Bact.* 72, 1956). A mixture of pantothenic acid (30–100 μg per ml.) and complex natural extracts (peptones) have now been found to replace the agar extract. The ash of peptone was not active. A number of the common vitamins, amino acids, and nucleic acid hydrolysis products failed to replace the complex extracts as a source of "methanica" (M) factor. A quantitative assay has been developed. A survey of natural materials indicated that M factor is widely distributed; also, it is synthesized by microorganisms. A rich convenient source is commercial (Wilson) liver extracts. Factor M was not destroyed by heating at 120° C. for 15 minutes at neutrality or in 1N NaOH or 1N HCl. Its behavior on ion exchange columns indicate factor M is strongly cationic. Purification studies are being continued.

POLIOENCEPHALITIS COMPLICATED BY TUBERCULOSIS AND INTRATRACHEAL HEMORRHAGE

JEROME O. RAVEL*

In recent years, tracheotomy has become an accepted procedure in a wide range of medical situations. Holinger *et al.*, (1) state that tracheotomy is an important part of treatment of respiratory obstruction caused not only by acute and chronic laryngeal inflammatory disease such as acute laryngotracheobronchitis and diphtheria, but also in poliomyelitis, cerbrovascular accidents, barbiturate poisoning, skull and face fractures, recurring postoperative atelectasis, tetanus, botulism, burns, bilateral laryngeal paralysis, and compressing mediastinal lesions. In poliomyelitis the indications for performing tracheotomy have been broadened through the years to the point where a rapidly failing vital capacity is considered a good indication. Although at first sight this suggests merely intercostal weakness, it also implies an ineffective cough and therefore an inability to remove secretions in a patient who will probably need respirator care. Furthermore, any patient who is desperately ill and must be nursed in a respirator should receive a tracheotomy even though other specific indications are absent (2,3,4,5). A case is presented in which inability to remove secretions by coughing followed by paralysis of respiratory muscles was considered an indication for tracheotomy.

Case Report

A 38-year-old male teacher of political science was admitted to Brackenridge Hospital, Austin, Texas, on June 16, 1955, with the chief complaint of weakness. The patient became ill on June 14, 1955, when he noted fever and generalized aching. He had back pain with stiffness of the right side of his neck. He was examined at home on the evening of onset when no nuchal rigidity was present and neurological examination was normal. Positive physical findings were T. 101° with sibilant rales over both lung fields. He was started on tetracycline and symptomatic therapy, and he was reported to

* Address: 1103 B Nueces St., Austin, Texas. Received for publication July 17, 1956.

be improved the following day, his neck no longer stiff. On the day of admission, weakness of the left arm was reported and increasingly severe cough was a prominent complaint. Low grade temperature elevation continued. The patient was examined at 5 P.M., and muscular weakness was not considered to be definitely present. However, the patient became extremely weak, nauseated, vomited, and was hospitalized later that evening.

Difficulty in hearing had become progressively worse during the past seven years. There was no exposure to tuberculosis. The patient had survey chest x-rays done repeatedly, and the last one in the spring of 1954 was negative.

Physical examination: T. 99°, P. 88, R. 24, B.P. 122/80. The patient appeared acutely ill, but he was alert and well oriented.

H. and N.: Defective hearing.

Lungs: Numerous sibilant rales over both lung fields.

C. V.: Regular rhythm.

Neuromuscular: Questionable slight nuchal rigidity was noted. Left deltoid weakness was present. Respiratory muscles were functioning normally.

Lumbar puncture was done and clear spinal fluid under normal pressure was obtained. A specimen sent to the laboratory for examination revealed cell count of 240 with 87 per cent lymphs; 11 segs.; 2 R.B.C. Spinal fluid protein, sugar, chloride—normal. Admission blood count: Hgb. 15.55 gms. or 107 per cent; R.B.C. 5.34 M.; W.B.C. 11,000 with segs.–70; stabs–2; lymphs–13; monos.–5. Urinalysis: specific gravity 1.019; microscopic: negative; sugar: negative; albumin: very faint trace. A diagnosis of poliomyelitis was made.

On the morning after admission, June 17, 1955, the patient complained that he had an uncomfortable night because of severe back pain. He had no fever during the morning, but dry rales persisted over both lung fields. The neurological findings revealed a decrease in muscle power in left deltoid and slight decrease in the left arm, forearm, and hand. Slight nuchal rigidity was present. However, the patient was breathing and swallowing without difficulty. He continued to be restless all day with intractable back pain which did not respond to aspirin, codeine, demerol, or hot packs. Tetracycline therapy was continued because of respiratory infection.

On the morning of June 18, 1955, intercostal paralysis was present. The patient was using his accessory muscles of respiration and abdominal muscles to breathe. The diaphragms were not moving and slight cyanosis was noted. The patient had been coughing frequently, but was unable to cough up secretions. E. N. T. consultation was obtained to have a tracheotomy performed, and patient was placed

in tank respirator. Weakness of the left lower extremity was noted that afternoon. Oxygen exchange in the respirator was satisfactory. T. 102.4° (R).

On June 19, 1955, he remained able to swallow and talk. The left arm was very weak, and both legs were paretic with the left weaker than the right. Patient was considered to be generally improved from the preceding day. T. 100–100.8° (R).

Early on the morning of June 20, 1955, the patient became psychotic and moved both arms in attempting to get out of the respirator. T. 103.4° (R). Blood chemistry: sodium 153 mEq./L.; potassium 6.1 mEq./L.; CO_2 combining power 62 volumes per cent; chloride 98.2 mEq./L. The organic psychosis was considered due to encephalitis and it continued during the next two days in spite of temperature diminishing to 100.6° (R).

Early in the morning of June 23, 1955, the patient was noted to be having episodes of cyanosis due to the tracheotomy tube slipping inside the respirator. When this was corrected, cyanosis disappeared. Parenteral fluids, vitamins, and antibiotics were continued. Maximum T. 101.4° (R).

On June 24, 1955, the patient was oriented in the morning and started taking fluids by mouth. T. 100.6° (R). However, he became disoriented again during the day and by 9:30 P.M., temperature had risen to 104.6° (R). It was impossible to determine whether pneumonia or secondary rise from polioencephalitis had caused the fever. Antipyretic measures were carried out, and antibiotic medication was changed from tetracycline to penicillin and dihydrostreptomycin. Sputum was sent to the laboratory to determine the predominant organism.

Temperature remained around 105° (R) for a day and gradually went down to 102°–103° (R) on June 25, 1955. Patient had episodes of cyanosis during the night although the respirator was working well and the airway patent. W.B.C. 21,150; segs.–72; stabs–25; lymphs.–1; monos.–2. He was oriented intermittently.

On June 26, 1955, temperature was 101°–102° (R). Bleeding skin around the tracheotomy tube was noted, but this was controlled by E. N. T. consultant while patient's breathing was maintained by an anesthetist, who reported the patient to be making respiratory efforts. Premature contractions were noted during the late afternoon. That night, auricular fibrillation set in, and on the following day cardiac rhythm was still completely irregular with the rate 120. Parenteral quinidine therapy was administered for twenty-four hours without change in the cardiac mechanism. Cedilanid D (8 cc.) was given intramuscularly, and two hours later the pulse was lowered to 75

to 80 per minute and was completely regular. There was no recurrence of mechanism disorder.

On June 27, 1955, W.B.C. 23,650; segs.–78; stabs–16; lymphs.–2; monos.–3; eos.–1. Blood chemistry: sodium 140 mEq./L.: potassium 6.0 mEq./.; CO_2 combining power 60 volumes per cent; chloride 90.7 mEq./L. Patient was given sodium chloride by mouth and parenterally because of the finding of low chlorides and low normal sodium.

On June 29, 1955, the patient was again disoriented, with slight fever. He had been kept in slight Trendelenburg position to promote respiratory tract drainage, and edema of the scalp was present. He was able to take fluids by mouth well, therefore none were given parenterally. Sputum was reported as being positive for acid-fast organisms, after two separate examinations were carried out on material aspirated from tracheal tube.

From June 30, 1955, to July 4, 1955, the patient was disoriented intermittently. He was taken out of the respirator for a few minutes at a time, and intercostal muscles were moving well. He was able to move his toes slightly, but the lower extremities were otherwise paralyzed and deep reflexes were absent.

On July 4, 1955, the patient was oriented while being changed to a Monaghan type of chest respirator uneventfully, but he became agitated and confused later in the day.

On July 5, 1955, W.B.C. 22,100; segs.–74; stabs–7; lymphs.–13; monos.–6. During the next several days, the patient was confused intermittently. Repeated smears for acid-fast organisms were positive, and were considered to be typical tubercle bacilli by a competent technician. He was allowed out of the respirator for eight hours without difficulty.

The morning of July 8, 1955, the patient was taken out of the respirator and had no respiratory difficulty until 3:45 P.M. when a large amount of blood suddenly appeared in the tracheotomy tube. The airway became obstructed, and he became cyanotic and lost consciousness. Pupils became dilated and fixed. Blood was suctioned from the airway and oxygen exchange was restored. Tracheotomy area was examined for possible bleeding source, but none was found. Bleeding stopped after patient lost consciousness. Cyanosis disappeared, and the patient had convulsive movements of the face and upper extremities. He regained consciousness completely at 9 P.M. and took liquids by mouth. Color was good and pulse was 80 and regular.

On July 9, 1955, the patient was irrational and agitated. A slight amount of bloody discharge was suctioned through tracheotomy

stoma. Bedside chest x-ray was reported to reveal parenchymal infiltration in both apices and first anterior anterspaces. The findings were considered to be in keeping with acid-fast involvement, and isoniazid and dihydrostreptomycin therapy were instituted, although a phthisiologist examined the patient and chest x-ray without observing a localized lesion which he expected to find as the source of the hemorrhage. He suggested erosion of a tuberculous tracheobronchial node through the mucosa as a possible cause of hemorrhage.

July 10, 1955, the patient again had a large amount of bleeding from the tracheal tube. In spite of two suction machines being used making constant suction possible, the airway filled with blood. The patient became cyanotic and pulseless before bleeding stopped. Epinephrine was injected into the heart and pulse became discernable. Airway was cleared by suction and blood transfusion started, resulting in shock being reversed over a period of approximately an hour. A bronchoscopy was done after exploration of the tracheotomy wound showed no bleeding point. Blood clots were removed by suction through the bronchoscope. During the bronchoscopy, blood was observed coming from the left upper main stem bronchus, and an ulcer, considered to be the probable source of the hemorrhage, was seen in that bronchus and cauterized with silver nitrate. The patient had another convulsive episode, regaining consciousness several hours later.

The day following the second bleeding episode, the patient was found to have lost eight years in orientation. He stated that he was 30 years old and in military service. Bloody mucus was suctioned from the trachea during the next few days, but there was no gross bleeding. He continued disoriented but did not live a specific past situation.

On July 12, 1955, laboratory studies revealed: Hgb. 14.5 gms.; R.B.C. 6.28 M.; W.B.C. 15,150; segs.–62; stabs–4; lymphs.–19; monos. –6; eos.–9. Bleeding time was 5′ 30″ and clotting time 4′ 50″. Clot retraction was complete in one hour. Platelet count 140,000 per cu.mm. Prothrombin time 100 per cent of normal.

On July 14, 1955, bleeding in the tracheotomy area was again noted but was not sufficient to obstruct the airway. Blood was suctioned from the tracheotomy tube and the trachea and bronchi were examined with a seven mm. bronchoscope through the tracheal stoma. The right bronchus was full of both old and fresh blood which was aspirated with a suction tip. There was a small amount of blood in the left bronchus. As the scope was removed, a spurting vessel was observed just outside the upper border of the tracheal stoma. The treacheotomy wound was reopened, enlarged vertically, and

bleeding vessels were sutured with chromic catgut to control the bleeding. The patient had no further bleeding or bloody discharge from the tracheotomy tube following this procedure. He was able to breathe well without the respirator, but was not weaned therefrom for several days.

On the night of July 16, 1955, the patient became oriented and continued so during the remainder of his hospital stay.

On July 18, 1955, the tracheotomy tube was removed, but the patient was unable to cough well enough to clear his tracheobronchial tree and the tracheotomy tube was reinserted. Physiotherapy for lower extremities was started, and the patient had gradual improvement of muscle strength. He continued to have positive sputum for acid-fast bacilli when material was suctioned from his trachea, with progressively fewer organisms found. Cultures were repeatedly positive for acid-fast organisms. Follow-up x-ray examination done on July 19, 1955, revealed the apices to be clear, and the radiologist commented that without the history of acid-fast etiology, as noted by laboratory studies, the chest would be considered entirely normal.

During the month of August, the patient was still unable to cough up bronchial secretion and the tracheotomy tube was left in place in order that suction of tracheobronchial tree could be carried out. Very gradual improvement of muscle strength in the lower extremities was noted.

On September 2, 1955, the patient was transferred to a Veterans Administration Hospital. The tracheotomy tube was removed a month after his discharge from Brackenridge Hospital and he was able to cough up secretions. He has gradually gained strength and resumed teaching in September, 1956.

Discussion

This polioencephalitic patient developed a series of complications following the onset of respiratory paralysis and disoriented state. Auricular fibrillation with no response to quinidine and dramatic response to cedilanid-D was noted. Myocardial involvement has been described repeatedly in severe and fatal cases of polioencephalitis (6,7,8), but specific mechanism disorders have received little attention.

An electrolyte imbalance with edema developed during the acute illness when the patient was found to have low chlorides and a low normal sodium level. The salt-wasting syndrome has been described in a group of patients with a variety of cerebral

lesions. The very nature of the cerebral disease which was diffuse in each instance (diffuse encephalitis, severe cerebrovascular disease, and bulbar poliomyelitis) defied any attempt to analyze the site of the responsible lesion. The syndrome was characterized by an inability to eliminate salt from the urine despite the depressed concentrations of sodium and chloride in the serum. These patients presented the evidences of true salt depletion, characterized by the retention of nitrogen and decrease in blood pressure, and responded favorably to the administration of salt (9). It has been suggested that upset in adrenocorticotrophic hormone production may effect the salt-retaining hormone from the adrenal cortex or that there may be a direct nervous influence from the central nervous system on the kidney that can modify the tubular reabsorption of salt (10). Another possible explanation of this phenomenon was found in that compression of the neck of sitting subjects caused increased output of sodium and chloride. However, little or no effect was obtained by this method in recumbent subjects, and although this patient had compression of the neck by the respirator collar, he was kept in recumbent or Trendelenburg position and this explanation cannot be applied in this case (11). The edema and electrolyte imbalance cleared when the patient was able to take oral fluids and food.

The final and most harrowing complication suffered by this patient was near-fatal intratracheal hemorrhage occurring on two occasions. The diagnosis of tuberculosis had previously been made upon repeatedly finding acid-fast bacilli in material aspirated from the trachea. This material later was found to have positive cultures for acid-fast organisms that were considered to be tubercle bacilli. Following the initial episode of intratracheal hemorrhage with loss of consciousness, bedside chest x-ray was considered to show evidence of parenchymal acid-fast disease. This was not agreed upon by all observers, but the hemorrhage being from an acid-fast lesion in the bronchus or lung was considered probable by all observers. Following the episodes of severe hemorrhage, it was considered necessary to make every effort to localize the lesion from which the hemorrhage arose in order that excision of diseased area be accomplished if possible. Further study, including bronchoscopy, failed to reveal a definite resectable source of the hemorrhage. Fortunately, when bleeding began for the third time, it was controlled readily and the bleed-

ing source was found in the soft tissues of the neck and bleeding stopped.

Karl Schlaepfer reported a fatal hemorrhage following tracheotomy for laryngeal diphtheria in 1924. He described ulceration of the tracheal wall by the tracheotomy cannula as a possible cause of hemorrhage, and in his case a large artery or vein was eroded by necrotizing inflammation several days after the tracheotomy was performed (12). A second fatal case of hemorrhage was reported in 1951 in a bulbar poliomyelitis victim who at autopsy was found to have a perforation of the innominate artery at the bifurcation, with the tear extending into the right carotid artery and slightly into the subclavian artery. The hemorrhage occurred into the tracheotomy wound and was not due to tracheal erosion at the tip of the tracheotomy tube within the trachea, which has been described as the most common cause of hemorrhage in this situation (13). A third case of fatal homorrhage was reported in 1954 from the rupture of the left carotid artery into the tracheal lumen immediately below the site of the tracheotomy. The lesion was initiated by the lateral rotary movements of the tracheotomy tube causing the tracheal erosion by the upward thrust of the carina upon the tip of the tube. Had the tube been shorter or the site higher, no such movement could have been induced (14).

Conclusions

A near-fatal case of hemorrhage into a tracheotomy opening is reported in a patient markedly debilitated by poliencephalitis complicated by electrolyte imbalance and auricular fibrillation. There is no intention to discourage the use of tracheotomy but merely to point this out as a possible complication of a procedure that is being used for a wider variety of indications than in the past. The fact that the patient had respiratory tract tuberculosis obscured the etiology of the hemorrhage.

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TYROSINASE ACTIVITY IN THE CELLULAR FRACTIONS OF HARDING-PASSEY MELANOMA

C. H. Robertson, J. C. Bergendahl, and E. C. Adams*

The Harding-Passey mouse melanoma was shown to contain tyrosinase by Hogeboom and Adams (6), in 1942, although other mammalian tyrosinase enzymes have been known since the beginning of the century (4). Further attempts to isolate and purify this enzyme system are currently in progress in this laboratory (1). It was thought worthwhile to extend past observations in order to ascertain the intracellular localization of tyrosinase. Previous studies of this nature have been reported by Lerner *et al.* (7). The steps in the oxidation of tyrosine to melanin involve the uptake of five oxygen atoms and the system can be measured by the usual Warburg techniques (8, 9, 10, 11). Other methods of measuring tyrosinase activity were also used.

Methods

The fractionation of a 1:10 homogenate of Harding-Passey mouse melanoma was undertaken. The tumor was ground in 0.25 M. sucrose in a Serval Omnimixer and reground in a Potter Elvejhem homogenizer. This homogenate was fractionated according to Schneider (12, 13). The nuclear fraction was obtained by centrifugation at 600 g for ten minutes. This precipitate was washed by resuspension in the sucrose solution and recentrifugation. The supernatant solution was centrifuged for ten minutes at 8,500 g. This and subsequent operations were carried out in the Model L Spinco centrifuge. The precipitate was washed twice by resuspension in the 0.25 M. sucrose. The final precipitate was resuspended in an arbitrary amount of distilled water and was designated as the mitochondrial fraction.

The supernatant fluid was then centrifuged at 18,000 g for one hour. The precipitate was washed once, resuspended in distilled water and designated as the microsomal fraction. The remaining

* From the Department of Biochemistry, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

supernatant fluid in the 0.25 M. sucrose was dialyzed against distilled water and the volume brought down to 100 ml. in a flash evaporator.

Half of each fraction obtained above was dialyzed for four days against distilled water, followed by dialysis against an ion-free water for the specific purpose of removing loosely bound copper. Copper is a component of the enzyme system and one of the purposes of this study was to determine the effect, if any, of dialysis on the system.

Aliquots from the original solutions and the dialyzed solutions were pipetted on to weighted aluminum planchets and the material air and then oven dried. These determinations were carried out in duplicate. Kjehldahl nitrogen determinations were done in duplicate or triplicate on the various fractions.

The tyrosinase activity was determined in several ways. A Warburg determination of the oxygen uptake of the homogenates was performed in a buffered phosphate solution at pH 6.8 containing a 3,4-dihydroxyphenylalanine, tyrosine substrate (4). The increment of O_2 uptake per ten minute interval is fairly constant between the 20, 30, 40 and 50 minute monometer readings and a mean of these values was taken as a more accurate estimate than the determination of the slope of a tangent to the microliters O_2 vs. time curve. These values are calculated as microliters of O_2/min. per mg. nitrogen and also as microliters O_2/min. per mg. dry weight.

A second method developed by Brown and Cooper was used (2). This consists of incubating small aliquotts of the fractions in holes on an agar plate containing 4 per cent 3,4-dihydroxyphenylalanine (dopa). A black ring is formed if there is tyrosinase activity. The amount of activity is roughly proportional to the size of the ring. In this experiment the results were expressed by numeral ratings from the most active, No. 1, to less active No. 2, etc.

A third determination was performed using a procedure developed by Fitzpatrick (5). In this method the various fractions were placed on stainless steel planchets which had been weighed on a micro balance. The planchets with the samples were then incubated for 24 hours at 37° C. in a phosphate buffer at pH 6.8 containing tyrosine-2-C^{14}. The radioactive amino acid was taken up and metabolized to melanin or melanin precursors. Subse-

quently when the planchets were rinsed in running water, any unreacted tyrosine was removed. The planchets were then vacuum dried, counted in a windowless flow counter and reweighed on the micro balance. These results are relative and expressed as counts per min. per 100 gamma dry weight of tissue.

Results and Discussion

The weight of material per ml., the total weight, and the mg. N per ml. are reported in table 1. There is a considerable loss in the weight of all fractions on dialysis. Part of this is a loss of the sucrose remaining in the fractions. The extreme loss of the whole homogenate on dialysis is due to the removal of the sucrose as this fraction was left in 0.25 M. sucrose while the others had been brought up to the stated volumes with water.

TABLE 1

Weights and Volumes of the Particulate Fractions

Fraction	Total Vol. ml.	Vol. dialyzed ml.	Vol. remaining ml.	Dry wt. mg./ml.	Total wt. gm.	Mg. N/ ml.	Dry wt. mg./ml. dialyzed	Mg. N/ ml. dialyzed
Whole homog.	26	12	12	96.0	2.51	1.97	11.0	1.88
Nuclei	100	49	49	28.4	2.84	1.88	16.5	1.03
Mitochondria	50	24	24	53.6	2.68	2.57	18.0	2.29
Microsomes	70	34	34	14.8	1.04	1.60	9.5	0.70
Supernatant dialyzed	100	100	..	...	3.04	...	30.4	1.98

Table 2 gives the results of the tyrosinase activities in the various fractions as determined by the three different techniques. It may be noted that there is little concentration of tyrosinase activity in any individual fraction. The nuclei show the least activity and the mitochondria the most. Microscopic examination of the fractions shows some smaller particulates and an occasional whole cell in the nuclear fraction. In the mitochondrial fraction there were a few nuclei. The necrosomae and supernatant fractions were free of nuclei, though some mitochondrial sized particles may have been present.

A comparison of the original vs. the dialyzed fractions reveals a gain of activity in all fractions when measured on a weight basis. There is also a gain of activity in all fractions except the whole homogenate when expressed on a mg. nitrogen basis.

There is an absolute loss of activity as shown by the Brown-Cooper method and by the absolute amount of O_2 taken up. This indicates a slight loss of activity in dialysis but a greater loss of inert material. It appears that at the pH of the dialysis there is no appreciable loss of copper or other dialyzable cofactors necessary for the activity of the enzyme system since the dialysis effects are minor. This could support the hypothesis that copper is firmly bound to the enzyme system. In the technique of Fitzpatrick there was a considerable loss of material from the weighed planchets and in some cases, the small weights of material left, led to uncertainty in the calculations. This type of error is considerably more marked with such homogenized suspended material than with merely ground or sliced sections for which the procedure was really designed.

TABLE 2

Tyrosinase Activity of Particulate Fractions

	Warburg			Fitzpatrick	Brown
Fraction	Microliter 0_2/min.	Microliter 0_2/min. mg. dry wt.	Microliter 0_2/min. mg. N	cts./min. 100 gamma	Rating
Whole homog.	4.35	0.046	2.25	3370	1
Nuclei	2.70	0.095	0.72	3450	9
Mitochondria	5.28	0.098	2.06	6830	4
Microsomes	3.70	0.130	1.16	5140	6
Supernatant dialyzed	4.46	0.074	1.13	873	2
Whole homog. dialyzed	2.89	0.218	1.28	3220	5
Mitochondria dialyzed	6.22	0.346	2.72	1530	3
Microsomes dialyzed	2.65	0.140	1.89	4530	7

The rating method with the Brown-Cooper agar plates is rather crude but gives a rough indication of relative activity.

A comparison of total activity from the various fractions with a calculation based on the activity of the whole homogenate accounted for approximately nine-tenths of the activity in the various fractions. This again is an evidence that there is no loss of cofactors necessary for activity during either dialysis or fractionation. The loss in activity on dialysis may mean that the molecule of tyrosinase is small and hence partially dialyzable. This confirms the work done by F. C. Brown (3).

These results are in partial conflict with previous ones in which the mitochondria appeared much more active as compared to the other fractions and in which there was a loss of activity on dialysis. This activity was restored by adding 10^{-3}M. copper. The amount of material used in this previous experiment was not carefully measured, and the copper, added in a 10^{-3}M. cencentration, is present almost in autocatylitic amounts, so that these preliminary conclusions now appear invalid. This previous fractionation was done in 0.88 M. sucrose.

Summary

A fractionation of Harding-Passey mouse melanoma into particulates was performed.

The tyrosinase activity was found in all the fractions with only a slight difference among them.

Dialysis of the fractions decreased the tyrosinase activity, but when results are calculated on a per mg. dry weight or a mg. N basis the tyrosinase activity appears increased.

The results yield some evidence that the tyrosinase enzyme is probably a small molecule, since a portion of the activity is lost on dialysis.

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EFFECT OF GLUTAMINE ON IQ SCORES OF MENTALLY DEFICIENT CHILDREN

LORENE L. ROGERS AND RICHARD B. PELTON*

More than a decade ago the possibility that glutamic acid might be effective in raising intelligence levels of the mentally deficient was noted when it was observed that children receiving 10 to 20 grams of this substance daily for *petit mal* epilepsy showed improvement in personality and mental acuity (5, 13). A long series of experiments designed to evaluate the usefulness of glutamic acid in improving mental function followed this early observation, but the results have been conflicting and inconclusive. The fact remained, however, that some individuals did show significant gains on glutamic acid supplementation. These studies have been adequately summarized in a number of review articles (1, 14, 15).

Recent evidence suggests that the closely related compound glutamine may play a much more specific and significant role in metabolism than has been generally recognized. In our laboratories glutamine has been shown to reverse the inhibition of growth of microorganisms caused by the addition of small quantities of ethanol to the medium (6). Further, a definite relationship has been established in animals between glutamine administration and voluntary alcohol consumption (7, 8). In neither case could the effects produced by glutamine be duplicated with glutamic acid. Preliminary trials indicate that glutamine may be an effective agent in the prevention of alcoholism in some individuals (9, 10, 12).

Tower (11), using tissue slices of human cerebral cortex, has demonstrated that glutamine is able to reverse the failure of epileptogenic slices to bind acetylcholine whereas glutamic acid is ineffective under the same conditions. Subsequent clinical studies by the same investigator (11) showed that intravenously

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administered glutamine affects the electroencephalographic activity of seizure patients by suppressing abnormally slow frequencies or by promoting a shift to higher frequencies. Studying the nutritional needs of two different cell lines grown in tissue culture, Eagle (3) observed that while glutamine supported the growth of both lines of cells, glutamic acid at any concentration failed to support growth of one type and permitted growth of the other only at concentrations 10 to 20 times that of glutamine.

Since all of these data point to an essential metabolic role for glutamine, an attempt was made to determine its effect on intelligence levels of a group of mentally deficient children with no history of epileptic seizures.

Experimental

Thirty children from the Austin State School served as subjects for the investigation. In order to avoid the complicating effects of various disease states, only those individuals for whom the etiology was recorded as familial or indeterminate were included. The children ranged in age from nine to fifteen years and in IQ level from 42 to 71.

Cattell's Test of G: Culture Free, No. 1, was administered individually to each subject by one of us (R.B.P.). The children were then divided on the basis of sex, age, and their test scores into three groups of ten each, with each group consisting of six boys and four girls. The children of Group I had an average age of 12 years, 1 month and an average IQ of 56.1, those of Group II an average age of 12 years, 4 months and an average IQ of 55.4, and those of Group III an average age of 12 years, 6 months and an average IQ of 56.6.

At midmorning of every weekday during the following 6-week period, each subject of Group I was administered one gram of L-glutamine dissolved in a half pint of chocolate milk, those of Group II were administered one gram of L-glutamine plus 25 mg. of pyridoxine dissolved in a half pint of chocolate milk, and those in Group III were given a half pint of chocolate milk to serve as controls.

At the end of the 6-week period Cattel's Test of G: Culture Free, No. 1, was again administered to each child by the same tester. Neither the tester nor the school officials had any knowledge of how the children were grouped or of which ones served as experimental subjects and which controls.

The scores made on the test before the supplementation period and after were then tabulated for each child. The results are presented in Table 1. A statistical analysis of the data was made.

Discussion

The results of this preliminary investigation are highly encouraging. In contrast to many studies carried out with glutamic acid in which 10 to 40 grams of the compound were administered

TABLE 1

Effect of Glutamine on Test Performance of Mentally Deficient Children

	Subject	Sex	Age Yr.	Age Mo.	IQ rating on 1st test	IQ rating on 2nd test	Change in IQ rating
	EDR	M	9	8	67	81	+14
	JNJ	M	11	5	43	54	+11
Group I	AEH	M	11	10	58	63	+ 5
(1 g. glutamine/day)	JDS	M	12	1	49	47	— 2
	CA	M	11	8	52	55	+ 3
	JB	M	10	8	45	54	+ 9
	GE	F	10	1	66	73	+ 7
	OJL	F	14	6	57	65	+ 8
	RNW	F	13	0	61	69	+ 8
	CFF	F	15	9	63	62	— 1
	Mean		12	1	56.1	62.3	+ 6.2
	RGS	M	11	1	64	74	+10
	EBR	M	9	8	68	69	+ 1
Group II	JB	M	14	2	55	58	+ 3
(1 g. glutamine +	FEF	M	13	1	54	64	+10
25 mg. pyridoxine/day)	KB	M	12	11	49	59	+10
	CEF	M	15	3	65	65	0
	WK	F	13	8	46	59	+13
	ER	F	9	2	49	55	+ 6
	LKS	F	12	3	53	55	+ 2
	HRN	F	12	5	51	56	+ 5
	Mean		12	4	55.4	61.4	+ 6.0
	JDD	M	14	2	57	65	+ 8
	JA	M	9	4	71	75	+ 4
Group III	RMB	M	10	0	51	47	— 4
(controls)	PEC	M	13	7	68	69	+ 1
	EML	M	12	2	44	37	— 7
	JNS	M	10	4	42	39	— 3
	MJB	F	12	5	56	65	+ 9
	NS	F	14	1	56	69	+13
	JP	F	14	8	60	60	0
	MN	F	14	3	61	63	+ 2
	Mean		12	6	56.6	58.9	+ 2.3

Statistical comparison of Groups I and II with Group III gives a P value of 0.95.

each day for periods of six to ten months, dosage in this investigation was limited to one gram daily for a period of only six weeks. Such restrictions were imposed because of the limited availability of glutamine at the time the experiment was planned. The data in Table 1 show, however, that even in this short experimental period, both groups of subjects receiving glutamine exhibited a much greater increase in IQ level than did those of the control group. The effect of any learning process involved was eliminated

by the design of the experiment. The experimental and control groups received identical treatment except for the administration of the supplements to the former. Therefore, any difference in the performance of these groups on the second test can logically be attributed to the effect of the supplements.

Because vitamin B_6 is known to participate in amino acid metabolism, pyridoxine was given along with glutamine to the subjects of Group II in an effort to determine whether such a combination might be more effective than glutamine alone. The results show that there was no significant difference between the mean increase in IQ rating of this group and that of the group receiving glutamine alone, the increment being 6.0 points for one group and 6.2 points for the other. When the data for these 2 groups are combined and compared with the data for the control group (for which the increment was only 2.3 points), statistical analysis reveals the difference in increase in IQ level to be significant at about the 5 per cent confidence level ($t = 2.03$).

No attempt was made in this study to obtain subjective evaluations of the subjects by trained personnel; however, the voluntary comments of the attendants in charge of the buildings may be of some interest. JDS, Group I, had been so emotionally disturbed by certain family difficulties that he had been eating and sleeping very poorly. The attendants were positive in their assertions that whatever he was being given had helped him tremendously. They reported that he not only ate and slept much better during the supplementation period, but that he appeared to be happier. There was also agreement in the reports that CEF, Group II, had shown improvement in personality. He reportedly got along better with those around him and paid more attention to his personal appearance than formerly. Surprisingly, neither of these boys showed any improvement as measured by the Cattell test. It is impossible to say at this point whether such reported improvements are the result of glutamine administration, but such effects, if real, might prove to be as important and valuable as increase in IQ level.

As might have been expected (16, 17), some individuals showed a much greater improvement in scoring ability after glutamine supplementation than did others. In the group receiving glutamine alone, the change in IQ scores ranged from an increase of 14 points for one child to a decrease of 2 points for

another. No child on glutamine plus pyridoxine made a lower score after supplementation than before, but several showed only slight improvement in contrast to the much greater improvement shown by others in the same group.

Some of the most interesting data from this study concerns EDR, Group I, and EBR, Group II. These subjects are twin brothers, though not identical. The school records showed each to have an IQ of 70 as measured by the Stanford-Binet test. On the first Cattell test EDR scored 67 and EBR scored 68, indicating that they were very close to each other in ability. On the second Cattell test, EDR scored 81, an increase of 14 points, while EBR scored 69, an increase of only 1 point. As a check on these results, the same test was administered two days later, at which time EDR scored 93 and EBR scored 73, Such divergence in scoring seems to indicate that a real improvement in mental ability had been brought about in one of these subjects and not in the other. This is further evidence for the highly individualistic biochemical make-up of even such close relatives as these.

The data presented here make it appear probable that glutamine supplementation is more effective in younger children than in older ones. The mean increase in IQ score for the five youngest children receiving glutamine was 7.4 points in contrast to an increase on only 4.6 points for the five oldest children in the experimental group. However, calculated on a body weight basis, the dosage used was considerably smaller for the oldest children than for the youngest, and the possibility remains that size of dosage is a critical factor.

A rigorous interpretation of these results must await further investigation, but a possible explanation may be found in the phenomenon of partial genetic blocks (2, 4). If it is assumed for the moment that some types of mental deficiency are associated with an impaired ability to synthesize glutamine from glutamic acid, then realtively small quantities of glutamine might be expected to be fully as effective as the much larger amounts of glutamic acid which have been used. In an individual in whom this reaction was only partially impaired, large amounts of glutamic acid could show an effect through being transformed—though inefficiently—into glutamine. If in other individuals the reaction was completely blocked—or nearly so—then even very large amounts of glutamic acid would cause no improvement but

relatively small amounts of glutamine might show an effect.

An alternative possibility is that the utilization of glutamine is impaired through a partial block of some reaction farther along the metabolic pathway. The individual variations in this study could be explained on this basis, especially data such as that obtained on the twins. The difference in ability of these two subjects to utilize glutamine might be great enough that one gram daily supplied the needs of one child but not of the other. In the latter case, larger doses of glutamine might be effective.

While the data presented here are too limited to justify the widespread use of glutamine in the treatment of mental deficiency at this time, they do warrant investigation on a much larger scale of the effectiveness of this compound. Such an investigation is now being planned.

Summary

Glutamine has been shown to produce a significantly greater increase in intelligence quotients of mentally deficient children as measured by Cattell's Test of G than was shown by a similarly-treated control group. The results for the group receiving glutamine plus pyridoxine were not significantly different from those for the group receiving glutamine alone.

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INDIVIDUAL PROGNOSIS

FREDERIC R. STEARNS*

Adhering to the definitive dichotomy of medicine as an art and as a science, it is consistent to consider prognosis as the main design of medical science; not only because diagnosis, treatment, and prevention aim at preconceiving the future fate of the human organism, but, more generally, because prognosis corresponds with the very meaning of science. Fremont-Smith (30) has concisely stated that the "whole basis of science is prediction and validation"; Lecomte du Noüy stressed: "The aim of science is to foresee and not to understand as so often has been said. Science describes facts, objects, and phenomena minutely, and tries to form then what we call laws, so as to be able to predict the future"(32). And more specifically (33): "When we have experimentally observed that certain definite conditions are always followed by certain phenomena which seem invariably linked to the first by a relation of cause and effect, we word this observation in such a way that enables us to predict these phenomena quantitatively and qualitatively whenever the same conditions are present."

It has long been recognized that all scientific laws, to which du Noüy is referring, rest on "chance," and that, therefore, the fundamental scientific method of prediction is the statistical method.

Statistics in medicine, as elsewhere, is scientifically applicable only to large homogeneous populations. Even conforming to this principle, the predictive statistical results are fraught with shortcomings. The fallacies of sampling and evaluation have frequently been pointed to (Berkson, J.; Snedecor, G. W.; Page, R.; Platt, R., and others); there is no need for repetition. It is more significant that, because of the peculiarity of biological material, errors in medical statistical evaluations are hardly avoidable. It is almost impossible, for instance to exclude concealed variables (80).

* Medical Director of the Security Benefit Life Insurance Company in Topeka, Kansas; Editor of Insurance Medicine, Louisville, Ky. Received for publication November 20, 1956.

Furthermore, "the incomplete manner" has been emphasized in which statistics represent reality (28), or, in other words, statistics applied to biological phenomena "never furnish an absolute answer" (58). Recently, Starr (136) has demonstrated that statistics cannot prove a cause-and-effect relationship and that the level of "significance" which is an essential factor in statistical presentations, "does not seem suited to many medical problems." In a nutchell, Myerson (104) has set forth the problematic role of statistics in medicine which cannot answer "why some people live longer than others" but, in a general way only show that, for example, "lean people live longer than fat ones." In his inimitable succinct fashion, Einstein (34) has epitomized the controversial position of precise measurement: "As far as the laws of mathematics refer to reality, they are not certain; and as far as they are certain, they do not refer to reality."

In spite of the inherent limitations of statistics and of the calculus of probability in medicine, their predictive value in group prognosis is incontestable (138, 139, 140). Insurance medicine and preventive medicine depend to a great extent on statistical evaluations of groups in order to predict morbidity, mortality, life expectancy, etc. A science of Public Health would be unthinkable without predictive statistical investigations on which measures of prevention can be founded. The main defect of statistical prognostic methods for the practice of medicine, is its limitation to groups. It is not relevant in individual diagnosis. Emil Borel has condensed this fact: "it will be always a meager consolation to think that the probability of misfortune was slight when he is the one to suffer from it." At best, as Hyman (77) has put it "in individual prognosis, statistics function as a weathervane."

Mathematicians, aware of this defect, have tried to introduce two concepts of probability. Russell (122), aside of the mathematical probability, admits a "degree of credibility which is the amount of credence that is rational to assign to a more or less uncertain proposition."

Jeffreys (79) has formulated a similar idea in stating that not all empirical situations can be appropriately described by the mathematical theory of chance phenomena, but the "theory of uncertain inference" may be suited which is an extension of traditional logic and which is a measure of the degree of "reason-

able" belief in some proposition in relation to a given body of evidence.

In most medical textbooks and clinical papers, prognosis is dealt with in two ways. One is reference to statistics and probabilities in groups of homogeneous or similar cases. Even if the frequent errors of sampling and evaluation are avoided, group probability is meaningless in the prognosis of the individual case. The other way is "the degree of credibility" or of "reasonable belief" which the physician or investigator has gained according to his experience and knowledge in individual cases or syndromes. Elsewhere (141), the confines of this partly empirical and partly intuitive approach have been studied and analyzed. The lack of proper methods was an evident fact.

A Short History of Individual and Group Prognosis

The part individual prognosis has played in the history of medicine reflects an increasing scientific neglect after the close of the Hippocratic era. It developed from a magic art in ancient oriental medicine to an equivalent of diagnostic empiricism in classic Greek medicine. With the dissolution of critical clinical observation, prognosis also became a victim of medical stagnation and mysticism in the early Middle Ages. At the end of that period and in the Renaissance, individual prognosis emerged as a casual accompaniment of the re-awakened empirical approach, but never resumed the essential role it had maintained in the Hippocratic school. Its significance languished because of lack of methodology; it became overshadowed by the differentiation of diagnosis and treatment. Finally, with the appearance of statistical methods in the 17th century which in the 18th century transgressed its sociological barriers and successfully invaded medical science, individual prognosis became a background phenomenon which borrowed its scientific light from the probability evaluation of group statistics.

From the Mesopotamian era (128), a "Book of Prognosis" (restored by Labat, R.) is preserved in which diagnosis was based on clinical observation 'a capite ad calcem' while the principal method of prognosis was divination, the interpretation of omens. In the Assyro-Babylonian period (43) prognosis was founded magically on liver inspection. In ancient Egyptian medicine—Berlin Papyrus 3038 (78)—a pregnancy prognosis test is re-

corded which roughly resembles the Aschheim-Zondek test. Hippocrates (14) in his book "Prognostikon" was the first physician to correlate clinical findings systematically with prognosis: "It is my opinion that the most excellent thing for the physician is to practice forecasting; hence, we must know the nature of these diseases how far they are superior to the bodily powers . . . one will indeed be better able to treat those capable of cure, if one has made up his mind long beforehand in each case, and no blame will attack him (the physician) if he has already foreseen and announced who is to die and who is to be preserved." The 'facies Hippocratica' as a prognostic sign of death is valid to the present time. The term glaucoma was used by Hippocrates for both glaucoma and cataract. "If the pupils are dark blue . . . and if it happens quickly and completely, then there is no hope for recovery. If the color is that of sea water, then the injury will develop steadily and very slowly and will seize the second eye a long time after the first." (75). It may be worthwhile mentioning that in the Hippocratic texts characteristics of the urine in diseases in connection with prognosis are alluded to more than 180 times. Aurelius Corneilius Celsus, in 30 A.D., recognized in nephritis (24) the prognostic value of measuring fluid intake and urine output, "for when the quantity of excreted fluid exceeds that which is ingested, then, at last, there is some hope of recovery." Aretaeus (about 150 A.D.), considering diphtheria, stated: "if it spreads to the thorax by the windpipe, it occasions death by suffocation within the space of a day" (5). Paulus Aegineta (625–690), writing on glaucoma, described the prognosis as always unfavorable (109). The Arabian physician, Albucasis (1013), stated about the prognosis of cancer: "As for me I never have been able to cure a case nor have I known of one who has." (2). The role of the urine as a prognostic tool, in the post-Hippocratic medicine, came to the fore probably first in Persia as the ritualistic procedure of uroscopy. In the 12th century, the Salernian physician Gills de Corbeil wrote a pamphlet on uroscopy. "Liber de Urinis." Later, these uroscopists were called "piss prophets" (63). Ugo Benzi (1376–1439), a remarkable medieval physician (93), distinguished himself by his emphasis on prognosis. He derived his interest in prognosis from Galen, and, particularly, from Aristotle's "De Divinatione in Somno," on which the scholastics and the Averroists had based their science of prognostica-

tion. In reading Benzi's case histories, his prognostic skill—partly empiric, partly mystic—appears surprising. In the 16th century, Ambrose Paré (1510–1590) wrote extensively on prognosis, which was based on clinical observations. "Great Aneurismas under the Arme pits, in the Groines . . . admit no cure, . . . Cataracts of plaister-like colour are usually incurable. On the contrary, such of chestnut colour, or of a skye or sea-water colour with some little whitenesse, yeeld great hope of a happy and successful cure" (106). Bernard of Gordon (1942) cautioned against a rash prognosis in cases of epilepsy, "since almost all epilepsy is eradicated with great difficulty if indeed it can be eradicated" (150).

In the 17th century, prognosis based on clinical observation, continued to some extent, but in the middle of that century statistics came into the focus.

As to individual prognosis. Thomas Sydenham (1624 to 1689) wrote in 1670 about the prognosis of measles: "the greatest danger arising from other diseases. especially lung conditions to which the patient may succumb." He pointed to the gravity of prognosis if the disease is of hemorrhagic character: "the measles are first livid, afterwards black" (147). Hermann Boerhaave (1688–1738) found the prognosis of lobar pneumonia favorable lowish with a little mixture of blood" (17). Henri François Le Dran (1685–1770)—stressed that the prognosis of cancer of the breast was unfavorable when the axillary lymph nodes were involved. Jean Louis Petit (1674–1750) corroborated this sign of poor prognosis and added the cervical and supraclavicular lymph nodes (113). Regarding the emergence of statistics in the 17th century, it was the pioneer work of John Graunt who, in 1664, published a book "Natural and Political Observations Upon the Bills of Mortality," in which he applied the statistical method on recorded figures. His work was enlarged by that of Sir William Petty (1623–1687) who, in addition to mortality statistics, collected data concerning rents, tithes, trades, religion, sexes, and diseases. In 1648 there was born in Breslau, Germany, a Caspar Neumann, who became a pastor, and who first tried to investigate the possible connection between human birth and the constellation of the planets. For this purpose he constructed tables from church records of deaths, for the years 1687 through 1690. He sent these tables in 1692 to the Royal Society in London which

commissioned the astronomer Edmund Halley (1656–1742) to study this material; Halley, in 1693, in the "Philosophical Transactions" published the first useful mortality table, using Neumann's data; he also calculated life expectancies from it. Blaise Pascal, in 1654, had already outlined the basic principles of the theory of probability (60, 1, 96, 14). These publications found the 18th century prepared for the abundant use of statistics and the probability calculus in medicine; individual prognosis declined progressively in significance.

Johann Peter Süssmilch (1707–1767), already influenced by statistical reasoning, emphasized that an inoculated individual is 25 times less likely to die from smallpox than one "who has not submitted to variolation" (146). As to the occurrence of rheumatic heart disease in the course of rheumatic fever, Jean Baptist Bouillard (1796–1818) formulated in 1836 his long famous 'law of coincidence'; "The coincidence is the rule and noncoincidence the exception." Sir Thomas Watson in 1843, however, limited this prognostic law by stating "that the younger the patient who suffers acute rheumatism, the more likely will he have rheumatic carditis" (115). These two examples, too, show how individual diagnosis tended to generalizations. Regarding statistics proper, it was the physician Pierre Alexandre Louis (1787–1872) who described the numerical method, later termed clinical statistics. In his thorough investigations on tuberculosis and typhoid fever, he tried to determine by statistical evaluations the main symptoms and the pathological lesions corresponding to them. He also made a statistical study on bloodletting, a main therapeutic procedure at his time, and proved that this panacea of François Joseph Victor Broussais (1772–1883), a leading clinician in Paris, in many cases was useless and even injurious. A contemporary of Louis, the Belgian Adolphe Quetelet (101, 116) (1796–1874) did statistical work in pediatrics. Since the middle of the 19th century, individual prognosis ceased to be a subject of systematic scientific research in medicine. Its situation was well characterized by Bloomfield (15): "Prognosis is based on past experiences; large accumulation of statistics, individual experience and laboratory tests are essential facts. . . ." The present state of irrelevancy of individual prognosis has been expressed by various authors. "The statistically oriented clinician makes his predictions warily in terms of his knowledge of probabilities in-

volved. The prediction of any particular event may not always come true as the atypical cases turn up, but at least such predictions are based on more valid assumptions than it is possible with any other system . . ." (155). And "until such time as more detailed methods for evaluation of single events become available . . ., the most valid alternative appears to be the assumption that any unique event lies along a normal distribution within a range of events the limits of which are known from clinical experience. . . ."However, the same authors immediately invalidate even this statement by stressing that prediction ultimately depends on the ability to control the variables present in an experiment; only if all variables can be controlled in a given experimental situation and if only one variable is varied at a time, a reasonably high degree of prediction can be expected (65). While this is hardly ever possible in a biological experiment which resembles reality, it is never possible in clinical conditions.

The main principles on which clinical medicine has been based, within the last 100 years, are differentiation of structure and function of the organism and specificity of clinical syndromes. Yet, Stewart Wolf has cautiously stated that "the question of specificity is not necessarily an essential consideration." W. Natrop (105), more stringently, stressed: "The doctrine of specificity is shaken." Hans Selye expressed a more eclectic opinion (125): "Nonspecific agents have been defined as stimuli 'which affect many targets and are devoid of the ability to act selectively upon any one.' By definition the reverse is true of specific agents. . . . The two appear to be quite unrelated. . . . Yet, even this fundamental difference between specific and non-specific is perhaps more apparent than real. It has been shown for a great many of reactive elements that they can respond to irritation only in one manner. Could it be that the apparent multiplicity of 'specific' reactions is merely due to combinations and permutations of such single reaction-type of which the various biologic elements of the body are capable? Should this be the case, then all manifestations of life, from the regional to the systemic, from the entirely non-specific to the most highly specific could be brought down to a common denominator."

Considering this trend to non-specificity, a brief remark may be in order on the newer evolution of the concept of disease. For if such a concept should overcome the present concept of differ-

entiation and specificity, individual prognosis, which has been thwarted by this working hypothesis, would also have a better chance to overtake diagnosis and therapy, methodologically.

A number of attempts have been made to base the concept of disease on a single denominator. The best known is that of the General Adaptation Syndrome and the Diseases of Adaptation by Hans Selye. He is concerned with the non-specific stressor actions which provoke non-specific defense measures, responses "whose protective stimulus is not limited to the stimulus which elicited them." Selye admitted that his work reemphasized the investigations by Ricker and by Speransky. Gustav Ricker (1870–1948) coined the term "Relationspathologie," stressing the interrelationship of all organic functions in all pathologic processes, and the fact that stimulation of the central nervous system is chronologically the first link in the chain causally connected physical processes in disease. A. D. Speransky (131, 132, 133), a pupil of Pavlov, placed the common denominator of disease in the central nervous system: "Neuralpathologie." The central nervous system is the starting point of physiological and pathological functions. By his experiments (freezing of the brain cortex with CO_2, removal and re-injection of spinal fluid, and pressure injury to the brain stem) he produced a 'periodically progressing neuro-dystrophy' often with a long latency period, and such varying reactions as alveolar pyorrhea, papillomas, hemorrhagic infractions of lung, stomach and intestine. "The nervous system makes the organism an acting unit. . . . Disease is not disorganization or disharmony of normal functions, but the emergence of new, qualitatively different processes, whose consequences then are functional disturbances." In this country, L. L. Whyte (163) has emphasized that physical, chemical and mental processes are special cases of a common principle. The interrelation of functions leads to a hierarchy of some of the structures and functions, which is responsible for the adaptation to the environment. In pathological processes and aging the normalizing processes fail in sustaining the normal unity of the organism. Stambul (135) has considered disease to be the result of a disturbance of mechanisms controlling the exchange of metabolites between the effector cells and their environment. These controlling mechanisms are the autonomic nervous system, the en-

docrine system and the electrolytes of the surroundings. Other non-specific approaches to the disease concept have been set forth by R. Reilly in France, and by Wm. J. White and Julius Jensen in this country. In scanning the literature, a few scattered attempts can be detected in which individual prognosis is founded on the principle of non-specificity of disease. A Michael Reese research team (6), in 1945, presented an individual 'factor of probability of survival' in hemorrhage (in dogs), treated with blood transfusions. The factor is the product of the mean arterial pressure and the CO_2 content of the blood, determined 30 minutes after withdrawal of 25 per cent of the animal's blood volume. Dogs with a low factor did not survive hemorrhage alternating with blood transfusions. A French paper (89) pointed to the prognosis of a poor general functional state by use of an intradermal injection of 0.5 cc. of a 1% mercuric oxycyanide solution; extent and induration degree of the skin lesion observed for a week, increased with poor physical resistance. Mueller (102) devised a "performance-pulse-index" for the prediction of general physical fitness. Using a bicycle ergometer the pulse frequency is observed during performance at an effort increase of from one to 10 mkg/sec. Persons in which high physical fitness can be predicted show values between 1.5 and 2, those with poor predictive fitness, values above 5. Two other German investigators (90), also using the bicycle ergometer with automatically increasing performance measured the time which lapsed until the product of pulse fretquency and pulse pressure reached the value of 10,000. This method is predictive for the highest possible physical performance of an individual. Wiener (164, 165, 166) stated that autosensitization (forming antibodies against erythrocytes) may be a predictive factor for the development of a number of systemic diseases: "It remains to be seen whether or not the presence of auto-antibodies demonstrated with the aid of enzyme treated with red cells is a warning sign that in the future the patient may develop hemolytic anemia, lupus erythematosus, or some other clinical sequelae of autosensitization." (Thrombocytopenic purpura, rheumatoid arthritis, periarteritis nodosa, atypical verrucous endocarditis.) Buerger (20) demonstrated that a longer life expectancy could be prognosticated in individuals who showed a prevalence of tonus of the para-sympathetic part of the auto-

nomic system. He showed that very old persons evidence such a predominance which is known to counteract excessive wear and tear of the cardiovascular system. Hardy (62) pointed out that the postoperative course could be predicted by testing the individual with corticotropin. Prolonged absence of circulating eosinophiles and depression of the 17-ketosteroid excretion have been found to be signs of poor individual prognosis after surgery.

These few at random examples of non-specific individual prognostic methods (the question of their scientific validity is not subject matter of this paper) lead to prognostic procedures, related to specific diseases, found in the literature. There has been no systematic scientific advance since statistics started to predominate as the predictive method. Therefore, all that can be detected in the literature is widely dispersed and often buried under the superstructure of diagnostic and therapeutic considerations. The picture that results shows, of course, lack of integration, particularly as the number of individual prognostic methods found in the literature is surprisingly large. Again it should be emphasized that this paper is not concerned with testing the cogency of the methods enumerated. Its sole purpose is to record some striking individual prognostic methods uncovered by perusing the literature with no claim to completeness.

In the evolution of medical art and science there may be found, for some period of time, in certain syndromes, an overlapping of diagnosis and individual prognosis. This is the case when either the diagnostic differentiation has not advanced enough for a consistent therapeutic attempt, or when the treatment methods applied have not proved to be fruitful. There are still a number of syndromes in which, for the present, poor individual prognosis and diagnosis coincide (*e.g.*, rabies, erythermalgia, Huntington's chorea). This overlapping is fortuitous and transitory; progress in diagnosis and therapy will abolish this overlay as past development has confirmed (*e.g.*, plague, cholera, typhus, subacute endocarditis, diabetes mellitus). Yet, it is obvious that in specific diseases individual prognostic methods can be devised only when the diagnosis can be clearly established and when a consistent therapeutical approach is possible. The individual prognostic methods contained in the literature are clinical, physical, or laboratory methods.

Current Individual Prognostic Methods

Cardiovascular-renal Diseases

Electrocardiogram. In coronary artery disease, exercise electrocardiography is a prognostic tool. If the affected area, after a period of rest, shows apparent healing, exercise tracing will indicate a good prognosis by exhibiting a tracing parallel to the stabilized tracing; poor prognosis will be indicated by reverting to a more acute tracing (36). As electrocardiograms are made increasingly a part of routine study of presumably healthy persons, more and more tracings showing bundle branch block are found in individuals without any other evidence or cardiac abnormality. The presence of bundle branch block itself has no bearing on the prognosis which is determined by the severity of the underlying condition. Where bundle branch block is found electrocardiographically with no clinical evidence of heart disease, it is probable that the patient in youth has had myocarditis, now completely healed, and that the lesion was strategically so located as to produce discontinuity of one of the bundle branches, a statis lesion, with no effect on legevity, cardiac output or reserve capacity (8).

Ballistocardiogram. A conspicuously abnormal ballistocardiographic pattern in an apparently healthy individual appears to be an unfavorable long-term prognostic sign, as evidenced in a follow-up study of 90 clinically healthy individuals with abnormal ballistocardiograms; they belonged to the age group over forty years (137, 18).

Rheumatic Fever. C-reactive protein, so-called because of its capacity to form a precipitate with C-polysaccharide of pneumococci, appears in human serum in response to inflammatory stimuli. This reaction is not restricted to pneumococcic disease. The test is a sensitive gauge for the acute phase of rheumatic fever if other inflammatory processes can be excluded. C-reactive protein titers respond more promptly to the fluctuations of rheumatic activity than changes in the erythrocyte sedimentation rate. From a prognostic viewpoint, as C-reactive protein antiserum can be used for demonstration of minute quantities of C-reactive protein in the serum, the test is of great value in following and predicting the course of the disease. Persistence of C-

reactive protein during treatment, in spite of clinical improvement, indicates inadequate suppression of the inflammatory process, while disappearance of the protein from the serum confirms a good prognosis as to the therapeutic or spontaneous suppression of inflammatory activity (121, 144, 38).

Patent Ductus Arteriosus with Pulmonary Hypertension. Poor prognostic signs for successful surgical intervention are: extremely loud and split pulmonary second sounds, absence or variability of the continuous murmur, characteristic with patent ductus arteriosus, conspicuous growth retardation, moderate to marked heart enlargment, electrocardiographic evidence of right ventricular hypertrophy, minimal increase in pulmonary blood flow as calculated following heart catheterization (123).

Hypertension. As hypertension shows a hereditary trend susceptibility to hypertension may be predicted in normotensives with a prominent family history of hypertension when they show varieties of vascular reactivity and response to salt restriction which can be observed in hypertensives (152, 153, 154). A prognosis of a benign course of essential hypertension can be made in hypertensive patients who are "overtly" nervous, become red-faced during excitement, and whose blood pressure falls to normal during sleep (22, 124).

There is reasonably good evidence that an individual with vascular hyporeactivity (a low maximal diastolic blood pressure under stress) will not have essential hypertension within 10 or 20 years. Vascular hyperreactivity in persons with usually normal blood pressure might eventually produce permanent vascular changes and, as a result, a sustained type of essential hypertension. The conclusion is that hyperreactivity (a high maximal diastolic blood pressure under stress) is prognostically of some significance and "perhaps of crucial importance" in the natural history of essential hypertension (66). Many people who have periodic abnormal elevation of arterial blood pressure may be considered prognostically as "prehypertensives" and develop full blown hypertension as time passes (107).

The cold pressure test was evaluated in 1932 (67). After many evaluations, Hines (68, 69, 70), stated that its prognostic reliability was not firmly established but that it was a useful tool in detecting vascular hyperreactors who usually have normal blood pressure and little or no hypertensive changes in the ocular fundi,

but who are hyperreactors in respect to diastolic blood pressure. Selye (126) expressed a more positive opinion in stating that "hyperreactors are believed to possess a hypersensitive vasomotor system which renders them especially liable to subsequent development of essential hypertension." He considered the cold pressure test a valuable prognostic procedure. Smithwick (129) pointed out that it is possible by means of the cold pressure test to predict the "peak load" to which the cardiovascular system may be subjected in periods of stress. It has been used to caution against a favorable prognosis of hypertension when the blood pressure after months or years returns "spontaneously" to normal. These falls in blood pressure may occur after cerebral accidents and coronary occlusion, and progression of arteriolar changes have been reported in the presence of normal arterial blood pressure (111). As to the prognosis of hypertension after sympathectomy, Hines (66) considers the cold pressure test a good indicator. "The postoperative response with patients in supine position may give an idea whether the hypertension will return within a reasonably short period of time."

The determination of capillary fragility has been considered a prognostic sign in hypertension as to: the occurrence of retinal hemorrhage, vascular cerebral accidents, and even fatal outcome (55, 56).

Thrombosis. A test, based on the relationship of alimentary lipemia and blood coagulability can be applied as a prognostic procedure in diseases (*e.g.*, diabetes mellitus and leukemia) in which lipemia and thrombosis are frequent features. Blood coagulability increases after intake of a fatty meal if macroscopic lipemia develops. Blood of patients are tested for macroscopic lipemia and clotting time before and after ingestion of a meal containing 85 gm. of fat. Acceleration of clotting time will vary from 4 to 15 minutes. Individuals with high acceleration of clotting time after fat intake tend to have thrombosis, particularly when suffering from arteriosclerosis, diabetes mellitus, and previous mocardial infarction (40).

Coronary Artery Disease and Angina Pectoris. Gofman and collaborators have pointed to the prognostic significance of blood lipoproteins (S^f 0–12 or S^f 12–400 lipoproteins). The elevation of these lipoproteins is a prognostic sign for the subquent development of overt clinical coronary artery disease (82). Increased

lipoprotein levels mean increased rates of accumulation of coronary artery disease in the form of arteriosclerotic narrowing, and an increased risk of coronary heart disease. "Not only the higher the lipoprotein level, the higher is the coronary disease hazard, but also the longer such a high level persists, the worse is the prognosis." It is suggested to test males from 20 to 29 years of age. If the lipoprotein levels are found to be very low, it is highly unlikely that the individual will develop coronary disease early in life. If the lipoprotein levels are high in an apparently healthy person of this age group, the subject will present a steadily increasing hazard of coronary disease, unless the levels are reduced (47). In females, lipoprotein levels rise more slowly than in males (46). Abnormally high lipoprotein levels occur on a familial basis (149, 48). When elevated lipoprotein levels are present already in children, they represent the main substratum from which the victims of coronary heart disease are derived even as early as in the third decade of life. Arcus senilis is frequently found in families characterized by very high blood levels of the Standards S_f 0–12 lipoproteins. Arcus senilis, therefore, should be considered a signpost for the possible presence of elevated lipoproteins. In patients with angina pectoris the likelihood of developing myocardial infarction is significantly higher when the initial lipoprotein levels are elevated in comparison with those patients with angina pectoris whose lipoprotein levels are in the lower ranges. In patients with a past episode of myocardial infarction the likelihood of a subsequent recurrence is the higher, the higher the lipoprotein level (97). Gofman also devised an index of "coronary atherogenicity," an arithmetic expression of lipoprotein concentrations. "The prediction of degree of coronary arteriosclerosis and the relative likelihood of development of clinical coronary disease by means of the atherogenic index based upon lipoprotein measurements is not only in qualitative agreement with established clinical and pathologic experience, but also in very good quantitative agreement." Gofman summarized the individual prognostic potentialities as follows: "From one type of measurements, namely serum lipoprotein determination, it is now possible to predict for a particular individual of either sex and at any age, the quantitative outlook for the development of fatal coronary heart disease. The method employed is to measure the Standard S^f 0–12 and S^f 12–400 lipoprotein classes ultracen-

trifugally" (48, 151). A study aimed to "preselect coronary-prone individuals" (43) resulted in the findings that males are predominantly coronary-prone individuals, especially when they are endomorphic mesomorphs and hard-driving, goal directed individuals. It also was found that in the saliva of such individuals there was a faster rate of change (oxidation-reduction) per minute during the test of 30 minutes duration. Xanthomatosis has been considered to be a condition in which later development of coronary artery disease may be predicted. Three types of Xanthomatosis: xanthelasma, xanthoma tendinosum and xanthoma tuberosum, are all familial diseases and all associated with significant changes of lipoproteins of low specific weight; this is particularly true in xanthoma tendinosum (50).

Disease of the Heart Muscle. Fraenkel in 1906 recommended an intravenous trial injection of 0.25 mg. strophanthin to diagnose and prognosticate the functional condition of the heart muscle in patients with subjective heart complaints and nonspecific signs. This trial injection recently has been again advocated by European physicians. If, after the injection, respiration, physical performance and sleep improve, if increased diuresis occurs and the patient feels subjective relief, the condition is due to organic muscular involvement and the prognosis corresponds with this disorder. If, however, the trial injection does not result in the above outlined objective and subjective changes, the complaints are due to other conditions (functional; varices in case of leg edema, chronic bronchitis, emphysema, silicosis, in case of lung involvement; etc.) and the prognosis differs according to the basic condition (30).

Nephritis. After complete recovery from acute glomerulonephritis (permanent disappearance of albuminuria, casts and erythrocytes in urine), lasting immunity is almost invariably established, irrespective of the frequency and severity of subsequent streptococcal infections (94). If chronic glomerulonephritis is accompanied by progressive hypertension, the outlook for more than a few years is poor. When progressive cardiac insufficiency develops, return to a benign course is unusual. Presence of chocked discs, arterial changes, hemorrhages or exudates in the fundi, corroborates a fatal prognosis within about two years. Pericarditis is always a prognostic sign of early death. When urea clearance is reduced to minimal values and phenolsulfon-

phthalein excretion is down to zero, the prognosis can be measured in months (95). The normal creatinine values in the blood vary from 1 to 2 mg. per 100 cc. They are increased in conditions in which nonprotein nitrogen and urea are increased. When creatinine values are increased in chronic nephritis, the prognosis is considered to be serious (51).

Hemophilia. The severity of the bleeding tendency is related specificially to the quantitative deficiency of thromboplastinogen. Cases of hemophilia may be divided into severe cases (thromboplastinogen too low to be measured and clotting time over 25 minutes) and moderately severe cases (thromboplastinogen concentration, 0.5 per cent to 1 per cent and normal or slightly prolonged clotting time). As a fairly close relationship exists between the concentration of thromboplastinogen, the clotting time and the severity of bleeding, the thromboplastinogen concentration test is a prognostic indicator as to the severity of the disorder (117).

Polycythemia Vera. Resistance to radiophosphorus treatment indicates a severe form of the disease (involving granulocytic lines, metaplastic erythropoiesis, and myelopoiesis) and a poor individual prognosis (143).

Communicable Disease

Tuberculosis. In clearly established cases of pulmonary tuberculosis, the erythrocyte sedimentation rate test is a reliable prognostic method as to the progressiveness of the disease. Periodic performance of the test in the course of the disease is essential as fluctuations in the sedimentation rate are particularly helpful in the prognosis of the individual case (103, 19). Active cases of tuberculosis with high erythrocyte sedimentation rates tend to have low urine secretion of Vitamin C; therefore, the determination of Vitamin C excretion in the urine under controlled conditions has approximately the same prognostic significance as the erythrocyte sedimentation rate (35). The time relation between local infection with tuberculosis and dissemination is of great importance in prognosis. The greatest danger exists during the first year after infection. If the time of infection can be ascertained by history of exposure to an infected person or the conversion of tuberculin reaction from negative to positive, special

therapeutic measures which may increase the resistance can change the prognosis in a favorable way (85).

Leprosy. A reliable prognostic test is the Lepromin (Mitsuda) test; an intradermal injection of minute quantities of lepromin antigen produces a definite allergic reaction in certain patients with leprosy while it elicits no reaction in others. When the test is negative the patient shows no defense and the disease will progress. When it is positive, the individual possesses adequate defense against Myco. Leprae; if he is already affected, the disease will remain mild and the patient may live without mutilations of the severe type of lepromatous leprosy (99, 119).

Histoplasmosis. Complement fixing antibodies appear in the blood serum in about 90 per cent of cases. In serial tests a rising titer (the titer is related to the quality and derivation of antigen) is observed, reaching a maximum in 2 to 3 weeks; this points to a recently acquired active infection. The titer begins to decline after 4 to 8 months. A sustained titer is likely to be found in cases of pulmonary histoplasmosis reinfected. A low titer associated with severe symptoms appears to indicate a poor prognosis (72).

Brucellosis. Persistent temperature elevation is an unfavorable prognostic sign (158).

Typhus Fever (louse born). Bad prognostic signs are rise in nonprotein nitrogen in the blood, severe hypertension, development of profuse hemorrhagic or purpuric rash (159).

Rocky Mountain Spotted Fever. A sudden rise in the white cell count is usually a bad prognostic sign. The extent and severity of the cutaneous rash is of less prognostic importance than the intensity of cerebral and cardiovascular involvement (160).

Diphtheria. The prognosis is directly proportional to the day of the disease on which antitoxin was first administered; this prognostic rule holds true for the first 7 days (161).

Immunization. Immunology permits one to predict the reaction of the individual to a number of infective agents. Active immunization in its various forms justifies the prognosis of lasting immunity in diseases such as chickenpox, cholera, plague, small pox, tularemia, Rocky Mountain Spotted Fever, typhus fever, yellow fever, poliomyelitis; lasting immunity with rare exceptions may be predicted in measles, diptheria, mumps, scarlet fever, typhoid fever, rabies. On the other hand, little or no

immunity can be prognosticated in erysipelas, furunculosis, influenza, lobar pneumonia and common cold. After passive immunization, immunity can be predicted with considerable degree of confidence in tetanus, gaseous gangrene, cerebrospinal meningiti, and diptheria. Tests predicting the effective level of immunity in an individual aganst a specific infection are: the Schick test in diphtheria, the Dick test in scarlet fever (not equally reliable), and the response to smallpox vaccination (26, 127).

Malignant Disease

Proteolytic enzyme inhibitors in the blood provide a means for testing the effectiveness of cancer therapy. Variations in the relative concentrations of chymotrypsin inhibitor and rennin inhibitor in the serum of cancer patients appear to be influenced by the growth rate of the tumor. Unfavorable prognosis (rapid growth) is associated with high chymotrypsin inhibitor and low rennin inhibitor values. The relationship is reversed in favorable prognosis when the cancer has been controlled clinically (169).

Episodes of venous thrombosis or of recurrent migrating thrombophlebitis not attributable to associated causes (surgery, pregnancy, blood dyscrasias, thrombangiitis obliterans, prolonged bed rest, etc.) and not influenced by anticoagulant therapy, may point to the presence or the development of an obscure visceral carcinoma. The prognostic significance may be seen in the fact, that attacks of venous thrombosis or thrombophlebitis may antedate the symptoms of visceral carcinoma by months, especially if the carcinoma is located in the pancreas or the periphery of the lung (119, 170).

α_2 globulin concentration in the blood serum in patients with myeloid leukemia and Hodgkin's disease may be a prognostic indicator for the activity and advance of the disease (3, 162).

Measuring the cell size in vaginal smear in carcinoma of the cervix, aids in the prognosis of this malignancy. If the cornified and precornified cells show an increase in size during irradiation, this increase in size is proportional to the effectiveness of radiation therapy. Cell mesurement provides an inexpensive prognostic aid (53).

In mammary cancer the prognosis appears to be most favorable when the rate of growth (mitotic interval) is slowest (118).

In skin cancer the degree of skin pigmentation seems to be a predictive sign of the susceptibility to cancer. Skin pigmentation seems to be related to the susceptibility to the carcinogenic action of solar radiation and to carcinogenic products obtained by distillation of coal and petroleum. Thus, minimal pigmentation of the skin seems to be a predictive sign for the susceptibility to skin cancer when environmental or occupational factors coincide (142, 110).

Endocrine Diseases

Diabetes Mellitus. The probability that the individual diabetic will acquire any or all of the chronic complications (vascular disease—notably disease of the coronary arteries and of the arteries of the extremities—diabetic retinitis, intercapillary glomerulosclerosis, and the diabetic neuropathies) increases with the duration of the diabetes and bears a direct relationship to poor therapeutic control (31). Capillary fragility is a good prognostic index for the occurrence of retinal disease, yet, the onset may be delayed in the well-controlled diabetic as compared with the poorly controlled patient. The longer the duration of the life of a diabetic the more likely is the incidence of vascular complications. The only difference between the well and the poorly controlled individual is the time of onset, later in the former, sooner in the latter (27).

Diabetes insipidus. The prognosis of idiopathic diabetes insipidus, regardless of age, appears favorable, particularly when the disease shows a hereditary trend. Symptomatic diabetes insipidus associated with brain tumors, Parkinson's disease and xanthomatosis has to be differentiated prognostically from the idiopathic form; the prognosis depends on the underlying condition. Normal pregnancy and delivery can be expected in female patients; the disease may show signs of remission during pregnancy (16).

Myxedema. Cases of untreated and fully developed myxedema have a poor prognosis and their life expectancy will be shortened. The prognosis in adequately treated myxedema is favorable. Disability can be expected only in patients who omit their thyroid medication (52).

Toxic Goiter. Prognosis in adequately treated cases, showing remissions by treatment with iodides or antithyroidal drugs,

should remain guarded because of the remarkable capacity of the disease to persist for many years even though all its manifestations are suppressed; "drug induced latency" is an important prognostic consideration (52).

Addison's Disease (Chronic Adrenal Insufficiency). A favorable prognosis as to survival depends on the possibility and economical means of the patient to obtain supplementary whole adrenal extract or cortisone when needed. In many instances death can be attributed to a deficiency of whole adrenal extract at a time of increased stress (156). Also the physician's understanding of the disease and of the necessity for prompt and heroic therapeutic action will determine the prognosis (167).

Hyperparathyroidism (Osteitis fibrosa cystica-von Recklinghausen's disease). After successful surgical removal of the adenomatous or hyperplastic parathyroid glands (difficulty: inconstant position and occasional presence of thoracic glands), the prognosis is favorable: the negative calcium balance is converted into a postive one, with rapid changes of the involved bones (57).

Diseases of the Liver

Cirrhosis of the Liver. Signs of poor prognosis are ascites, prolongation of prothrombin time, anemia, hypoalbuminemia, and small size of the liver (23). In Laennec's cirrhosis, there is a close correlation between the level of serum albumin and the clinical status; a sustained rise in serum albumin is considered a favorable prognostic sign (108). The cephalin flocculation is very valuable in determining whether the cirrhotic process continues in spite of adequate treatment; if the cephalin flocculation reaction remains unchanged, the prognosis is unfavorable (61).

Collagen Diseases

Lupus Erythematosus. Transient presence of the L. E. cell phenomenon in patients with drug hypersensitivity, paralleling the self-limited disease, contrasts with the persistent L. E. cells and prolonged clinical course in patients with untreated, classical systemic lupus erythematosus. The "false positive tests" in patients with drug hypersensitivity might indicate the presence of acute acquired reversible lupus erythematosus, with an obvious etiology and a favorable prognosis. In idiopathic systemic lupus

erythematosus, which is an irreversible process, the prognosis is unfavorable (64). The course may be chronic with remissions and relapses. In patients who have discoid lesions only on the face and subsequently develop generalized lupus erythematosus, the prognosis as to the period of survival may be better; the duration of life from the onset of the localized skin lesions to death may be a decade or more. Such instances are rare (148, 168).

Diseases of the Nervous System

Nerve Injuries. Neurotization is the term applied to the recovery of nerve supply. The electromyograph provides information as to the degree of innervation and the condition of the muscle. The so-called tetanus-twitch ratio also is a valuable measure. Strong electric currents may cause tetanic muscular contractions while a threshold current may cause a brief twitch. In normal muscles the ratio is between 5.5 and 6, it approaches only 1 in denervated muscles, yet rises with regeneration. Electromyography and the (galvanic) tetanus-twitch ratio, used in conjunction, can provide sufficient information to predict further progress in neurotization (134, 114). Prognosis of the adaptive capacity in paralyses of certain muscles or muscle groups depends on the special muscle training of accessory muscles; for instance, training of the ulnar nerve in the apposition of the thumb to the fingers when the median nerve is paralyzed (98).

Brain Injury. The deeper and more permanent the disturbance of consciousness, the graver is the prognosis; irritability and a confused, dazed, or lethargic state are less serious prognostic signs. Changes in pupillary reflexes, bilateral Babinski, absence of superficial reflexes, sluggish deep reflexes, also are signs of unfavorable prognosis (100).

Myasthenia Gravis. The prognosis is not equally "gravis" in all cases. When the condition initially is ocular in distribution and remains so for two or three years, there is a good predictive chance that the disorder will remain local and will produce little disability (37).

Poliomyelitis. The ultimate state of muscular recovery depends on the amount of strength remaining in the paralyzed muscles at approximately 6 weeks. In making these predictions, it is essential to determine whether or not each muscle is paralyzed diffusely or in a spotty fashion; for, muscles with areas of

spotty paralysis show little or no recovery in these areas, making the prediction for the muscle as a whole inaccurate. A formula expressing the final recovery for diffusely paralyzed muscles, in percentages of normal and in terms of the 6-weeks status is:

$$L + \frac{100 - L}{100} \times \frac{10\,L}{\frac{1 + 9L}{100}}$$

L equals strength of muscle at 6-weeks expressed in percentage of normal.

This formula was derived from experimental evidence that arborization of an axon can occur sufficiently to produce at least 5 times as many nerve endings as it normally has and twofold hypertrophy may further strengthen the muscle. From this formula the prediction can be made that a muscle with 10 per cent of normal strength at 6-weeks will recover a final state of approximately 57 per cent of normal, while a muscle with a 40 per cent strength of normal at 6-weeks will attain approximately 92 per cent normality. It follows that most muscles which have no more than 60 per cent loss of strength at 6-weeks will return practically to normal function (59. Another author (13) pointed to a somewhat different predictable pattern of recovery of strength in individual muscles or muscle groups. By the end of the third month following onset of acute weakness, a muscle will usually recover 60 per cent of the total strength that it will ever recover under treatment. During the following three months an additional 20 per cent of muscular strength may be recovered; by the end of 18 months of treatment no further increase in muscle strength can be expected. Severity of muscular paralysis can be predicted to be greater in individuals who continue their usual physical activity or engage in vigorous exercises after appearance of meningeal signs (headache, vomiting, stiffness of neck) than in those who are kept at bed rest from the time of onset (25).

Epilepsy. An improvement in the electroencephalogram, as the child grows older, is a sign of favorable prognosis. A record that becomes more abnormal with the years indicates a poor prognosis. In the adult, an electroencephalographic record showing less abnormality through the years, greater stability of activation, and constancy of form and pattern, carries a far better prog-

nosis than a tracing which develops increasing abnormalities. (87). In persons with no overt convulsive disorders, disturbed brain wave potentials may indicate predisposition to epileptic seizures (87). "Febrile convulsions" in children probably present a mild form of inherited epilepsy; the prognosis is considered favorable in both febrile and non-febrile genetic epilepsy. This contrasts with a relatively unfavorable prognosis for various forms of organic epilepsy. The electroencephalographic patterns in febrile convulsions are generalized rather than focal, with spontaneous improvement as the brain matures (91).

Mental Diseases

Schizophrenic Reactions. Clinically, the only factor of predictive value is tension, a symptom which also predominates in affective and psychoneurotic reactions. It is justifiable to state that high physiologic reactivity to acute external stress is one of the cardinal features of cases with a favorable prognosis. It also has been suggested that prevalence of an epinephrine-like substance, characterized by relatively higher pulse rate and warmer face and hands at rest, would point to a better prognosis; so does, under conditions of stress, the greater fall in blood pressure with mecholyl and greater reactivity of the pulse to immersion in ice-water. In terms of the traditional division of the autonomic nervous system in sympathetic and parasympathetic parts, it would appear that patients with a favorable prognosis present constant high sympathetic tension (54). Other authors stressed that high lipid blood contents which are synonymous with an extroverted personality, are predictive for recovery (45). The Funkenstein test consists of injections of a sympathetic nervous stimulant, epinephrine hydrochloride, and of a parasympathetic stimulant, mecholyl hydrochloride. This test has been used to predict reactions to electroshock and insulin coma therapy. Characteristic blood pressure changes following the injections (decrease following mecholyl and increase following epinephrine) are said to be related to a favorable outcome of both kind of treatments. This study demonstrates that reactive sensitivity of the autonomic nervous system as indicated by blood pressure changes after injection of a cholinergic drug, is a valuable tool in the prognosis of behavioral changes and length of hospitalization (76). Another paper enlarges on the same tests. If a patient, schizophrenic

or not, has anxiety precipitated by epinephrine injection, he is not likely to improve with electroshock. If anxiety is precipitated by mecholyl injection, or if chills occur, he will usually improve with electroshock. One may predict a good therapeutical result of electroshock in cases: (a) in which epinephrine causes a marked rise in systolic blood pressure with return to the pre-injection level after 3 to 10 minutes: (b) in which mecholyl injection causes marked fall in systolic blood pressure with failure to return to the pre-injection level within 25 minutes; (c) in which chills occur after mecholyl injection; (d) in which anxiety is precipitated or relaxed by mecholyl injection. A poor therapeutic outcome is predicted in cases in which epinephrine precipitates anxiety (41).

Profrontal Lobotomy. It appears that mesomorphy is related to a favorable prognosis. It has been evidenced that if the patient, subjected to either lobotomy or topectomy, is not a mesomorph the prognostic outlook for a successful operation is by far less than in mesomorphs (86).

Involutional Psychosis. Prognosis in involutional depression can best be estimated by taking into account, in addition to the symptomatology, an external "socio-psychiatric" factor, and an internal psychological factor. The nearer the clinical picture approaches classical melancholia with retardation, self-reproach, etc., the better is the outlook. The more isolated the patient, the fewer the human contacts, the less favorable is the prognosis (21).

Geriatric Psychiatric Disorders. In a large group of patients over 60 years of age, it was found that individual prognostic signs for early death included coincidence of two or more of the following signs: male sex; age of 80 and over; focal nervous system lesions; absence of functional symptoms in organic psychosis. A favorable prognosis could be arrived at in patients with purely functional disorders (88).

Psychosomatic Medicine. In studying psychoanalytic material for the purpose of prediction of the hormonal status (estrogen and progesterone) by means of the vaginal smear methods, the hormonal prediction, based on the psychoanalytic material, was correct in the large majority of the individual cases (12).

Phenylpyruvic Oligophrenia. Aside of the family history, the hereditary metabolic aberration can be established by a positive

ferric chloride test in the urine for phenylpyruvic acid. If the test is positive, the prognosis is invariably unfavorable for mental development (81, 92).

Recovery from Mental Disease. Applying the Minnesota Multiphasic Personality Inventory, it is possible to predict the speed of recovery. Patients with delayed recovery from a variety of diseases had more abnormal profiles than those with normal recovery. With the same method it was possible to predict that patients with relatively normal profiles were better subjects for psychotherapy and would improve under shorter treatment than those with abnormal profiles (120).

Miscellaneous Conditions

Growth. Growth prediction has been founded on the accumulated knowledge of the human growth pattern as related to skeletal maturation; the maturity indicators are shape of the long bones, and size and shape of their respective epiphyses. Reproductions of hand X-ray pictures at successive 6-month intervals provide standards against which unknown X-ray pictures are matched. According to Bayley's prediction tables, the individual growth potential is prognosticated in taking into consideration chronological age, skeletal age, and present height. "The greater the degree of maturity either in years or in development, the less growth potential remains (11, 9, 10, 44, 157).

Obesity. An attempt was made to predict in obese patients unfavorable reaction to weight reduction program. The "night-eating syndrome," composed of evening hyperphagia, insomnia and morning anorexia, would make attempts at weight reduction remarkably unsuccessful and would serve as a prognostic indicator (145).

Genetics and Marriage. For genetic prognosis in individual marriages, individual characteristics and traits in the family history of the person tested are charted, the potential effects of the environment on a family trait are considered, and the environment favorable to the active development of a family trait is specified. Thus, "it is often possible to give accurate estimates of the chances of the reappearance of the character in a family" (130).

Resistance to Surgery. Eosinopenia of longer than normal duration after surgery appears to point to unfavorable individual

prognosis. Prolonged excessive secretion of mineral-ocorticoid hormones may be responsible for a fatal outcome (4).

Toxemia of Pregnancy. A combination of urine concentration test and urea clearance test gives satisfactory information regarding the prognosis of the individual case (29).

Serous Otitis Media. When the fluid evacuated during myringotomy is thick and contains eosinophiles, the prognosis was found to be less favorable than in cases in which the fluid was thin, yellow and serous (84).

Summary and Conclusions

Medical science whose essential subject, considering both the purpose of medicine and the definition of science, is prognosis, in the course of history of medicine has shifted emphasis from individual prognosis to group prognosis based on statistics and the calculus of probability. This direction is in agreement with the attainments of exact science. Yet, aside of the fact that group prognosis is not, or only in an indirect way, applicable to individual clinical cases, employment of the statistical method in biological material is fraught with inherent shortcomings which are inevitable even when the frequently encountered mistakes of sampling and evaluation can be avoided.

The problem arises whether a methodology of individual prognosis is possible, which in view of the prevailing opinion of specificity of disease, could elaborate differentiated procedures paralleling those of diagnosis and treatment. Perusal of the literature shows that scattered reports deal with methods of individual prognosis. While the examples enumerated in this paper certainly do not comprise the majority of such methods which could be detected in the entire medical literature, they present an instructive cross section. Although within the past century there was no integrated endeavor to establish differentiated procedures of individual prognosis, the examples at hand point to the practicability of developing such methods systematically.

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TENTH ANNUAL SYMPOSIUM ON FUNDAMENTAL CANCER RESEARCH

The University of Texas

M. D. Anderson Hospital and Tumor Institute

INTRODUCTORY REMARKS ON NUCLEIC ACID METABOLISM

(Presented in part before the morning session and in part before the afternoon session.)

DR. VAN R. POTTER

Professor of Oncology, The University of Wisconsin, McArdle Memorial Laboratory for Cancer Research

LADIES AND GENTLEMEN:

Today we shall hear some of the latest developments in the field of Nucleic Acid Metabolism. Before proceeding to this discussion, I want to state on behalf of myself and the members of the panel, that we regard it as a privilege to come here and to hear from each other and to hear your discussion. We have enjoyed very greatly the sessions that were held yesterday and we are very much impressed with the beautiful Institute here. So far we have greatly enjoyed our stay and I'm sure that we will continue to do so. For myself, I wish to thank the local people for permitting me to select the speakers on this panel. While there are many others working in the field of nucleic acid metabolism, that we would like to have with us, I think that we have here a group which you will enjoy hearing and the work that you will hear is being pursued at the fundamental level and with an eye toward practical chemotherapy. I think that you will find it hard to distinguish which papers contribute more to the practical side and which papers contribute more to the fundamental side because in this collection of papers you will find that the fundamental viewpoint has been stressed in working with chemotherapeutic agents, and I am sure that the studies which have not directly involved chemotherapeutic agents have had the practical aspects in mind. So it no longer is correct to think of applied versus fundamental research as mutually exclusive. We have here a collection of papers which make a deep contribution from both sides. This of course no longer needs to be emphasized because I think all scientists recognize that fact. Now it is customary for

the chairman to talk for five or ten minutes while the auditorium fills and so I will carry out that role. I think it is important to look at the overall picture of the role of nucleic acids. Since the 1860's nucleic acids have been with us, and the striking studies that were carried out some 20 years ago in terms of crystallization of the first enzymes and the first viruses has shown that the nucleic acids represent a fundamental unit of biology. I see Dr. Stanley in the audience and we are all very much aware of the importance of the preparation of the tobacco mosaic virus as a ribonucleoprotein in pure form and we are very much aware of the recent dramatic studies that have been carried out with this particular ribonucleoprotein. Since 1950 progress has been so rapid that concepts and ideas are everywhere rapidly building up to a fairly definitive picture. And today I believe that the time has come to present publicly a general formulation of life processes which in the present context serve as a background for the talks to come. This theory is based on a bringing together of many recent facts and aspects that represent views that are shared by many biochemists. Recent publications by Dr. C. P. Rhoads of the Sloan-Kettering Institute (I refer to a recent paper in *Science*), shows that people in cancer research are very much aware of the therapeutic possibilities that arise in connection with nucleic acid metabolism. Now all of us working in the field of cancer chemotherapy recognize the fact that the ultimate cure for cancer may well come from interference with a variety of metabolic pathways. We are not claiming that the sole path to glory lies down the nucleic acid highway, but this field is certainly on the march and I think it will be interesting to see today the documentation of what is being done and what can be done in this area. Now the basic theory that I wish to present is represented in figure 1 and I am sure that there will be those who will disagree about whether one should formulate such a concept in such definitive terms. It is frequently said that it is dangerous to give the impression that we know possibly more than we do. I hope I am aware of the limitations of the biochemist and I don't think for a minute that we will ever understand all of the intimate chemical details of life processes. However, I do confidently believe that at this moment in history we are in possession of enough facts to justify the presentation of a basic formulation of life processes and I don't think it is dangerous—I think it is neces-

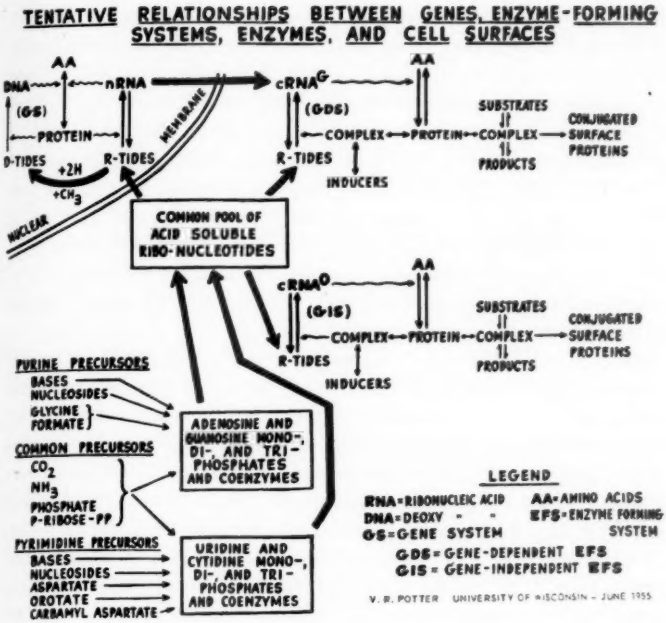

FIG. 1

sary. I think it is necessary to formulate concepts that can be placed all on one chart so that we can discuss them and so that we can see the interrelationship between the various facts at our disposal. From a biological standpoint, it has come to be accepted that genes somehow form the basis of enzyme-forming systems which are in turn responsible for the individual enzymes in tissues as indicated in figure 2. You are all aware of the 1:1 concept of gene to enzyme that is associated with the names of Beadle and Tatum, and the counter arguments that have been advanced.

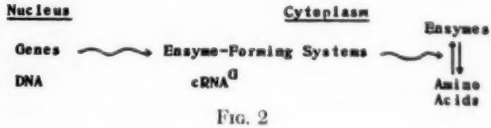

FIG. 2

More recently we have become aware that a given gene doesn't necessarily correspond to a given enzyme in terms of what one can observe in a whole cell. But our problem here is to translate these biological terms into the terms of the biochemist. The best approximation that we can make as of today, in 1956, is that if not identical, certainly the relationship is very close between deoxyribonucleic acid (DNA) on the one hand and the genes on the other hand. To be more specific, it would appear likely that one DNA molecule may be the bearer of a large number of genes, each of which is a pattern of nucleotides within the DNA molecule. And more and more evidence is piling up to suggest that the enzyme-forming system is ribonucleic acid in nature (Fig. 2). Now as this field has moved along we've become aware of the distinction between RNA in the nucleus and RNA in the cytoplasm, although it is still unclear as to whether cytoplasmic RNA could be formed independently of nuclear RNA or whether the nucleus was solely responsible. In figures 1–4 the small *c* represents cytoplasm and the small *n* represents the nucleus. From unpublished work in our own laboratory and from the literature I wish to propose the concept that there are two broad classes of RNA in the cytoplasm (Fig. 3). We put a superscript G with one and a superscript O with the other to indicate the concept that the RNA^G class has a corresponding template back in the nucleus in the form of DNA and that the RNA^O class does not have a corresponding template back in the nucleus. So we refer to RNA^G as the *gene dependent* enzyme-forming systems and RNA^O as the *gene independent* enzyme-forming systems. Then the important distinction that I wish to make has to do with the equilibrium constants for the raw materials for these nucleic acids and on the

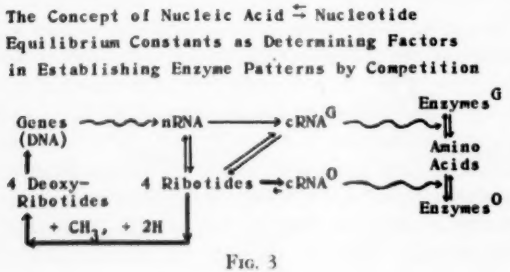

FIG. 3

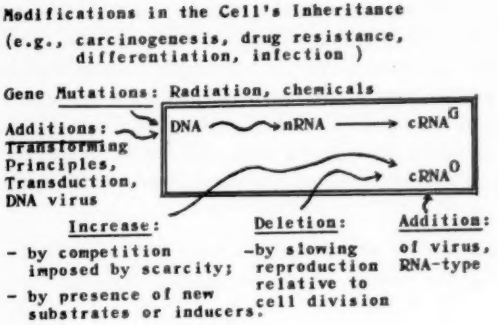

FIG. 4

basis of deduction from a variety of studies it seems to me highly probable that as a class, the RNA^G type of enzyme-forming system is in ready equilibrium with the ribotides. We are all aware of the beautiful work being done in Dr. Ochoa's laboratory which indicates that the specific ribotides that enter RNA are probably the diphosphates of the mononucleotides. The concept that I am presenting this morning is simply that the RNA^G systems, which are most directly related to DNA, are readily reversible and they can afford to be because their template back in the nucleus is formed by an essentially irreversible action. On the other hand there must be certain classes of ribonucleic acid molecules (RNA^O) in the cytoplasm which are essentially irreversible or very slightly reversible (Fig. 3). And finally there is the point that certain ribotides which are common to both these systems are actually convertible into the deoxyribotides which form the raw materials for DNA (Fig. 3).

We believe that the RNA^O elements can be looked upon as self-duplicating systems and that whenever the rate of multiplication of these systems falls below that of cell duplication, that the opportunity arises for losing the enzyme-forming systems of the RNA^O variety. The loss or deletion of one of the RNA^O elements, since it is not backed up by a nuclear gene, could result in an irreversible change in the cell. By this line of thought the changes in carcinogenesis could occur as the result of a virus infection which would represent an RNA system of the RNA^O type which would be able to compete very effectively with these RNA^G and

RNA^{0} systems and upset the balance between them. On the other hand mutations at the nuclear levels could be reflected into changes in the RNA^{G} elements, in fact the change from the RNA^{G} type of a system to the RNA^{0} type could result from a nuclear mutation or loss and according to this line of thought, carcinogenesis would be simply a special instance of the general mechanisms by which one line of cells is converted into another (Fig. 4).

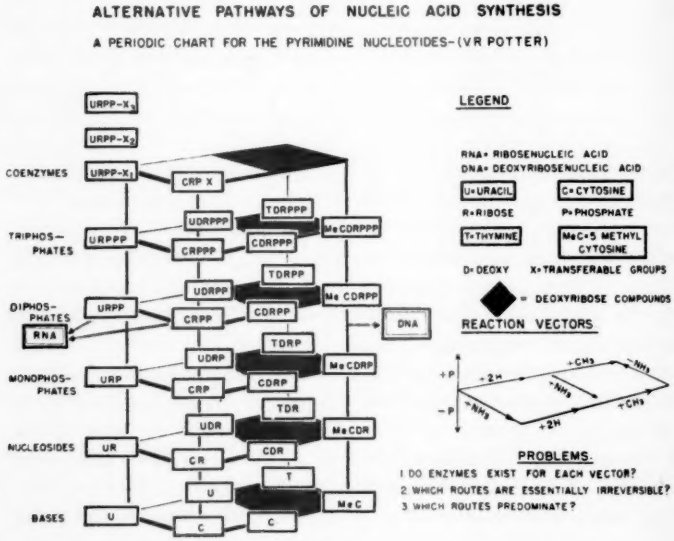

FIG. 5

Finally, the chart in figure 1 presents a resumé of the alternative pathways of synthesis for the nucleotides that form the building blocks for nucleic acids.

The extent to which figure 1 condenses the intermediate pathways between the distal precursors and the nucleic acids is revealed in part by figure 5 which indicates some of the possible pathways in the area of the pyrimidine nucleotides, which have been of particular interest in studies in our laboratory. There is little doubt that this chart will have to be revised considerably in the next few years, but it may be helpful in orienting some of the studies to be presented today. These pathways will be discussed in

much greater detail by the speakers on this program, who will also describe studies with blocking agents which interfere with various specific steps in the synthesis of the nucleic acids.

Now my time is up and I do not intend to discuss this further but I simply leave it to the audience to listen to the eight papers which will be presented today and to consider how these concepts are related to the research which is to be presented.

NUCLEIC ACID SYNTHESIS IN REGENERATING LIVER*

CYRUS P. BARNUM, CHRISTINE D. JARDETZKY
AND FRANZ HALBERG

When we ask ourselves—why does a cell divide?—we are likely to answer that certain conditions within the cell have become opportune for this process to occur. Perhaps the concentration of an essential metabolite or of some extracellular messenger has increased to a critical level or, alternatively, perhaps the concentration of some inhibitor, whether of intra- or extracellular origin, has decreased to a critical level. We must then raise the question to what extent this "critical state" has developed as a result of intracellular metabolism and to what extent its development was influenced by factors originating outside the cell. If we next focus our attention on the malignant cell we might conclude that the attainment of this "critical state," though not necessarily occurring more rapidly than in some normal tissues, is no longer under appropriate control of the organism. Somehow the malignant cell has lost its perspective and does not know when to stop. This unwillingness or inability to cease proliferating would appear to represent some altered pattern of metabolism, no longer integrated with the functions of the organism as a whole. It is from this point of view, that a better understanding of the pattern and of the controls of metabolism during normal growth may help us to understand the abnormalities of malignant growth.

The liver affords a very convenient tissue in which to study certain aspects of growth. Following removal of lobes amounting to about ⅔ of the liver mass the remaining lobes rapidly proliferate until the original mass is restored (16). In our hands, working with the mouse, this process starts about 30 hours after partial hepatectomy and is completed in about 5 weeks (19).

* From the Department of Physiological Chemistry and the Division of Cancer Biology of the Department of Physiology, University of Minnesota Medical School, Minneapolis, Minnesota. These investigations were supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, in part by a grant from Eli Lilly and Company, and in part by research funds from the Elsa U. Pardee Foundation.

Other investigators have reported about 70% restoration (8, 22) or essentially complete restoration (6, 16, 26) two weeks after partial hepatectomy. This restorative process, after an initial lag phase, achieves a maximum rate between the first and second day after operation and then continues at a diminishing rate until complete restoration is accomplished.

Liver in the process of restoration differs metabolically from normal adult liver in several respects but perhaps the most striking difference is in the area of the synthesis of DNA (deoxyribonucleic acid). In normal liver, as in other resting tissues, where few cells are preparing to divide, the incorporation of a labeled precursor, such as P^{32}, into DNA is extremely small compared to its incorporation into RNA (ribonucleic acid) or other constituents (3). On the other hand, in proliferating tissues, where new DNA must be synthesized, incorporation of P^{32} into DNA can be readily detected and measured (4, 7, 15). This suggests using the relative specific activity of DNA at any given time as an index of the rate of proliferation. Figure 1 depicts an experiment in which the incorporation of P^{32} into liver DNA was measured at various times after partial hepatectomy. At each point the mice were sacrificed one hour after the intraperitoneal injection of P^{32}. The DNA specific activity is reported as percent of the specific activity of inorganic phosphorus and the curve depicts, as a first approximation, the fraction of DNA synthesized per unit time at various stages during the restorative process (19).

The metabolic inertness of DNA in a resting cell is apparent not only from experiments showing the very low incorporation of labeled precursors but also from experiments showing the high degree of retention of a precursor that had been incorporated during a stage of rapid proliferation (7, 14, 19). Figure 2 depicts an experiment in which P^{32} was injected intraperitoneally into mice 36 hours after partial hepatectomy, i.e., at a time when its incorporation into DNA is rapid. Groups of these mice were sacrificed at various times after the operation. It may be seen that the specific activity of acid soluble phosphorus (ASP), which at early times after P^{32} injection was much higher than that of DNA, decreases rapidly to a very low value. Not only does ASP specific activity fall rapidly but from 2 days after P^{32} injection the specific activity of cytoplasmic phospholipid falls equally rapidly on a curve indistinguishable from that for ASP. Furthermore, in a

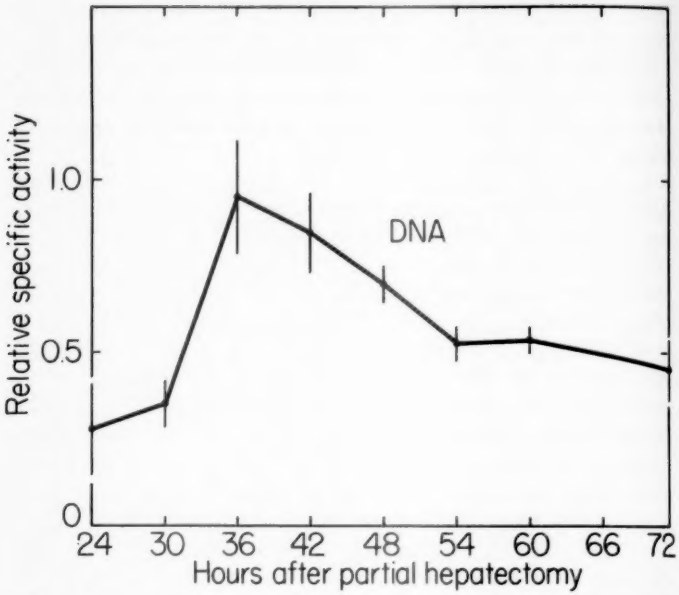

FIG. 1. DNA specific activity as percent of inorganic phosphorus specific activity at various times after partial hepatectomy. P^{32} injected intraperitoneally one hour before sacrifice. Vertical lines in this and subsequent figures each indicate ± one standard error of the means.

terminal group of mice (not shown in Fig. 2) sacrificed 10 weeks after operation the total cytoplasmic phosphorus (ASP, RNA-P, lipid P and protein P) had a specific activity a little lower than that of ASP at 8 weeks. The specific activity of DNA shows a decreasing trend up to about 5 weeks after operation, i.e., up to the time when restoration of the original mass is complete. This fall in DNA specific activity is to be expected since the originally labeled DNA is being diluted by newly synthesized DNA of lower specific activity. However, from 5 weeks on, when the livers may be considered as essentially in a resting state again. the DNA specific activity remains high and at 8 weeks is about 50 times the specific activity of ASP.

Referring again to figure 1 it may be seen that the one-hour relative specific activity of DNA seemed to vary least in the neighborhood of the value obtained at 60 hours after partial hep-

atectomy. The period from 58 to 64 hours after operation was selected for use in a kinetic study. For the kinetic study all mice were subjected to partial hepatectomy between 9 and 11 P.M. and all were sacrificed between 8 A.M. and 2 P.M. at varying times after injection of P^{32}. At sacrifice the livers were fractionated into nuclear, microsomal, ultramicrosomal and supernatant fractions and the nucleic acids of these fractions prepared as previously described (4). Figures 3, 4 and 5 show the specific activity-time curves for inorganic phosphorus (IP) and for the phosphorus of DNA, nuclear RNA (NRNA), supernatant RNA (SRNA), ultramicrosomal RNA (URNA) and microsomal RNA (MRNA). In confirmation of the observations of a number of investigators (3, 20, 23) the NRNA shows a much more rapid incorporation of labeled precursor than do the cytoplasmic RNA's. Also the 3 cytoplasmic RNA fractions studied can be differentiated on the basis of their specific activity-time curves.*

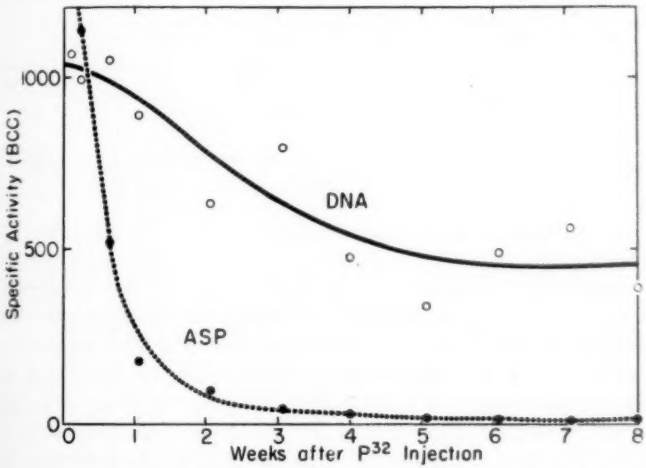

FIG. 2. Retention of P^{32} in DNA of regenerating liver. P^{32} injected 36 hours after partial hepatectomy. Mice sacrificed at various times after injection. BCC (Biological concentration coefficient) equals

$$\frac{\text{counts/min./millimole P}}{\text{counts/min. injected/gram body weight}} \times 100.$$

*A fuller discussion of these results including a mathematical evaluation of possible precursor-product relationships will be reported later.

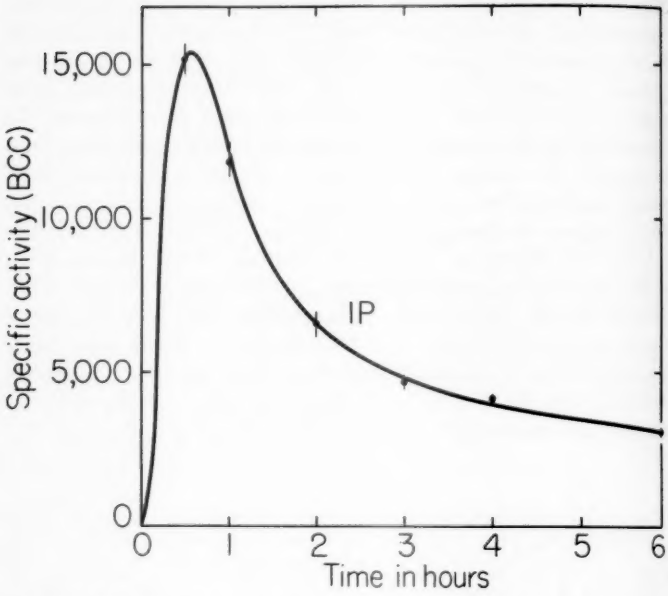

FIG. 3. Specific activity-time curve for inorganic phosphorus in regenerating liver.

As can best be observed for NRNA in figure 4, but is true for the other nucleic acid fractions as well, the specific activity-time curves of the various nucleic acids tend to converge toward the curve for inorganic phosphorus. One means of comparing nucleic acid metabolism in tissues of varying growth rates is to plot the rates at which this equilibration with the specific activity of inorganic phosphorus is taking place. In figure 6 the specific activities of NRNA, MRNA and DNA in 3 tissues at various times after P^{32} injection are plotted as percent of the corresponding inorganic phosphorus specific activties. The tissues are a transplantable mouse mammary carcinoma (T) having a growth rate of 2 to 2.6% per hour (4), regenerating mouse liver (R.L.) 58 to 64 hours following partial hepatectomy and having a growth rate of the order of 1% per hour (19), and normal adult mouse liver tissue (N.L.) (3). It may be seen that the more rapid the growth rate the more rapid the rate of equilibration for all three nucleic

acids considered. With respect to NRNA, however, this equilibration is occurring very rapidly even in normal liver whereas for DNA the rate of equilibration appears to be roughly proportional to the growth rate of the tissue. It should be borne in mind that a given fraction, such as NRNA, is isolated from all the cells in the tissue under consideration and that the specific activity observed is the average specific activity. Thus while the average rate of equilibration of NRNA with inorganic phosphorus is only about 50% greater in regenerating liver than in normal liver, the rate of this process in that fraction of the cells that are engaged in net synthesis of NRNA might be very much greater.

These observations that various nucleic acid fractions from growing tissues incorporate precursors more rapidly than similar fractions from a resting tissue still do not answer the question as to how this condition was brought about or in what manner the organism might be able to control the establishment within a cell of the "critical state" necessary for duplication of that cell.

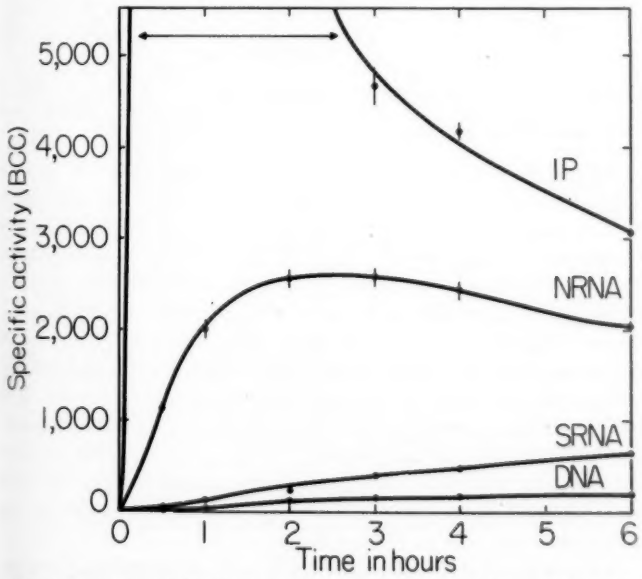

FIG. 4. Specific activity-time curves for inorganic phosphorus (IP), nuclear ribonucleic acid (NRNA), supernatant ribonucleic acid (SRNA), and DNA in regenerating liver.

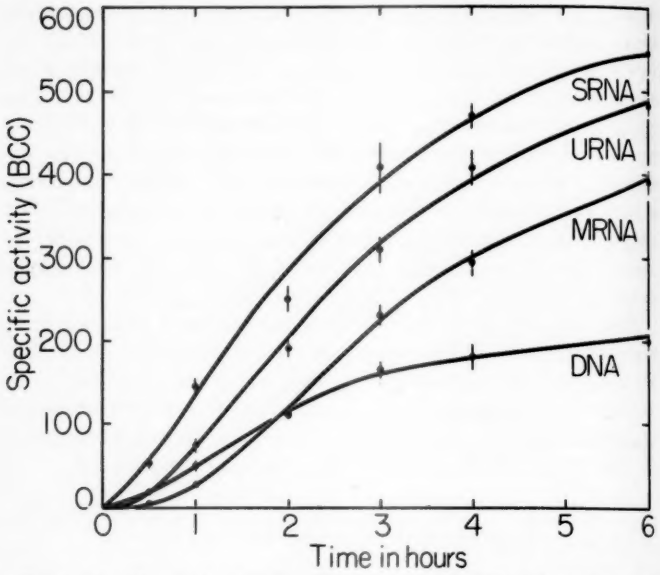

FIG. 5. Specific activity-time curves for supernatant RNA, ultramicrosomal RNA, microsomal RNA, and DNA in regenerating liver. (For possible limitation to assumption of constant rates see text below and figure 8.)

The fact that P^{32} incorporation into DNA appears to occur only in cells that are preparing to divide, coupled with the well documented observations of daily periodicity in mitotic counts (for review see 9), suggested a critical study of 24-hour periodicity in DNA synthesis as evidenced by isotope incorporation. Such a study appeared promising in view of a reference by Kelly *et al.* (21) to diurnal variations in DNA metabolism and in view of 24-hour periodic variations in microsomal RNA and phospholipid metabolism previously observed in this laboratory (2,10). It is also pertinent to mention that the flat portion of the DNA specific activity-time curve in figure 5 is consistent with the assumption that the rate of DNA synthesis was lower during the later times of the kinetic study (11 A.M. to 2 P.M.) than during the earlier intervals (8 A.M. to 11 A.M.).

The projected study was aimed not only at evaluating possible periodic fluctuations in isotope incorporation into DNA but also at correlating such observations with the mitotic activity of the

tissue at various times of day. Mitotic periodicity characterizes histogenetically different tissues (13) of various mammals in the immature and mature states as well (9). Furthermore, as demonstrated by Blumenfeld (5) in 1943, mitotic periodicity is characteristic of regenerating as well as normal epithelium of the rabbit. Recently Jaffe (18) noted a striking daily periodicity in mitotic activity in the regenerating rat liver with high activity in the morning and low in the evening. Thus it would appear that regenerating cells retain their marked potential for proliferation over a period of several days but also that the body is able to exert a controlling influence on this process. An experiment was therefore set up in an attempt to detect such controlling daily periodic effects on DNA metabolism and their relation to mitotic periodicity in regenerating liver.

The experimental circumstances of the periodicity-phase of this work on DNA and mitotic activity were standardized as has been done for previous work on other 24-hour periodic variables (9, 11). The animal room was illuminated between 6:00

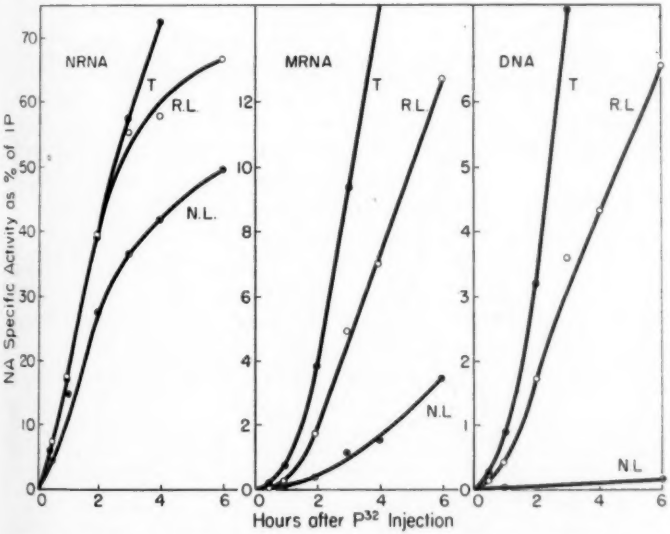

FIG. 6. Rates of equilibration of specific activities of NRNA, MRNA and DNA with IP in a transplantable mammary carcinoma (T), regenerating liver (R.L.), and normal liver (N.L.).

A.M. and 6:00 P.M., was kept dark from 6:00 P.M. to 6:00 A.M. and was maintained at 78 ± 1° F. The operations were performed on the same day within a 3½ hour period on mice that had been singly housed for at least seven days in the animal room just described. 180 mice were subjected to partial hepatectomy under nembutal anesthesia between 7:00 and 10:30 A.M. and after the operation were returned to their individual cages in the animal room. As in the previous experiments with regenerating liver, these mice were given 10% dextrose in their water bottles and were allowed Purina Fox Chow *ad libitum*. 140 of those mice surviving the operation had rectal temperatures at the times of sacrifice that were within the range of the 24-hour temperature rhythm evaluated on comparable mice in the same laboratory. From this group of 140 animals 14 were selected for sacrifice at each of 10 times following operation. Two hours prior to sacrifice P^{32} was injected intraperitoneally. At the time of sacrifice sections were obtained for histological examination from each regenerating liver and the remainder of the liver was used for isolation of ASP and DNA.

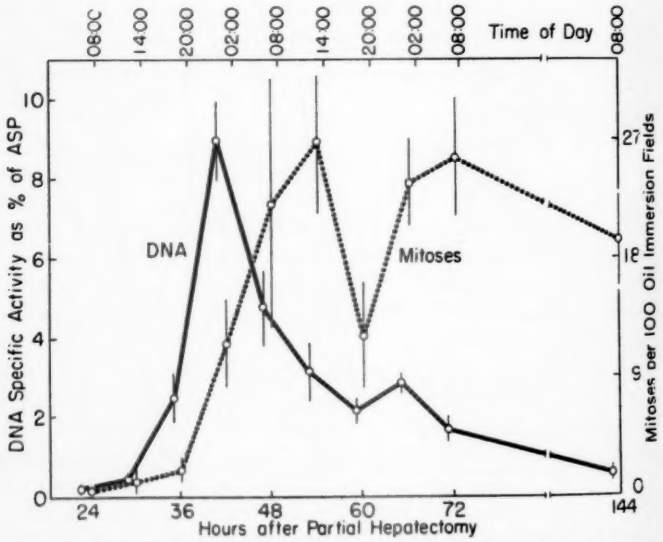

FIG. 7. Periodicity in DNA relative specific activity and in mitosis of regenerating liver. P^{32} injected intraperitoneally two hours before sacrifice.

In figure 7 the results are plotted by recording DNA specific activity as percent of ASP specific activity and by recording the number of mitoses per 100 oil immersion fields. In comparing figure 7 with figure 1 it may be seen that by performing all operations at a given time of day on a carefully standardized group of animals it has been possible to detect a second peak in the DNA curve occurring 24 hours after the primary peak ($P < .05$). It may be seen that mitotic activity also shows a cyclic variation throughout the course of the day with a peak about 2 P.M. and a trough about 8 P.M.*

Thus it would appear that both mitotic activity and DNA synthesis in regenerating liver undergo a periodic daily variation. By inspection of figure 7 it seems safe to conclude that, in regenerating liver, mitosis follows DNA synthesis by a period of 6 to 12 hours. It may be seen that the mitotic curve shows its first marked rise 6 hours after a similar rise in the DNA curve and that the peak in the mitotic curve occurs 12 hours after the primary peak in the DNA curve. These data on time relations of DNA synthesis and mitosis in regenerating mammalian liver (Mus musculus) seem to be roughly in agreement with related data on plants; namely with those of Howard and Pelc (17) on bean root tips (Vicia faba) and also with the data of Taylor and McMaster (25) on the tissues of lily (Lilium longiflorum) (for discussion, see 25). But an exact definition of the time interval between DNA synthesis and mitosis in regenerating liver must await data obtained at shorter intervals.

Moreover, in regenerating liver the analysis of the daily periodicity in the relative specific activity of DNA *per se* is complicated by a superimposed longer-term trend. The shape of the DNA curve in figure 7 represents, probably, the interaction of trend and periodicity. While indeed the effects of both trend and periodicity are interpreted as significant, the former effect may have interfered with the latter. If this assuption be valid, the 24-

* These observations of 24-hour periodicity in mitotic behavior after partial hepatectomy may be regarded as being qualitatively similar to Jaffe's observations. However, any attempt to compare the two sets of observations quantitatively would not seem to be justified because of differences in experimental procedure. For example, Jaffe used rats, under environmental circumstances that were probably not strictly comparable to those of this investigation, and he administered colchicine 4 hours before sacrifice. With respect to this last difference Mühlemann, *et al.* (24) have recently discussed the effect of colchicine on mitotic periodicity.

hour periodicity in relative specific activity of DNA will be more prominent in the absence of an interfering trend. Indeed, this same periodicity stands out with unmasked clarity in an appropriately standardized study of the livers in normal young mice.

140 ZBC male mice between 31 and 33 days of age were standardized as mentioned above. Two hours after injection of P^{32} they were sacrificed in groups of 20 every 4 hours beginning at 8:30 A.M. one day and ending at 8:30 A.M. the next. At sacrifice aliquots of the liver homogenates were taken for glycogen and from the remainder ASP, DNA and MRNA were isolated for evaluation of their specific activities.

The specific activity of ASP showed very small fluctuations throughout the 24 hour period but the relative specific activities of DNA and MRNA, i.e., their specific activities as percent of ASP specific activity, showed marked and highly significant variations throughout the day as may be seen in figure 8. A striking periodicity was found also in liver glycogen which dropped at night to little more than 1/10 of its morning value. This finding is qualitatively in keeping with earlier work on the same subject (for pertinent references, see 2). The timing of the daily peak in liver glycogen content and its absolute value will depend largely upon feeding. But timers or synchronizers of periodicity (11, 12) must be distinguished from intrinsic controlling mechanisms (9). The assumption that factors other than feeding also are operative in the maintenance of glycogen periodicity is in keeping with the demonstration by Agren, Wilander, and Jorpes (1) who observed such a periodicity in fasted animals.

Another interesting feature seen in figure 8 is the observation that the curves for relative specific activity of DNA and MRNA are out of phase. The relative specific activity of MRNA is rising at a time when that of DNA is falling. The physiologic significance of leads or lags in phase of related metabolic events seems to deserve further study.

Whatever the mechanisms involved may be, these significant periodic fluctuations in nucleic acid synthesis and/or metabolism constitute a characteristic of immature growing as well as of regenerating liver. The findings imply a degree of control on the part of the organism over the attainment of a "critical state" at the cellular level. The proliferating liver in the process of restora-

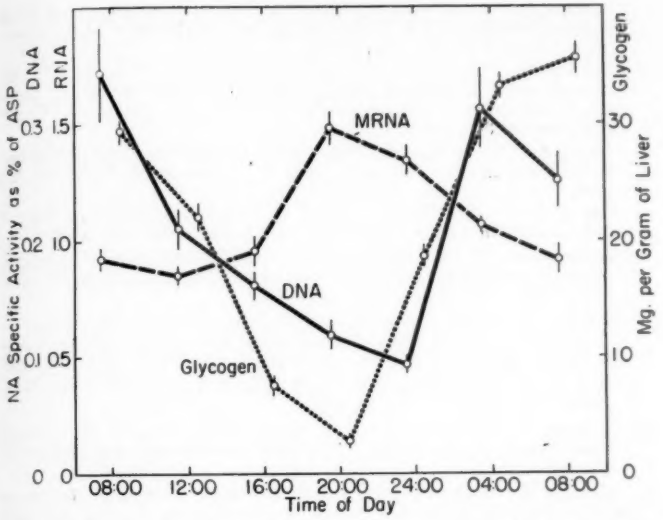

FIG. 8. Physiologic daily periodicity in relative specific activity of nucleic acids in immature liver—in the process of normal growth. P^{32} injected two hours before sacrifice. Corresponding periodic changes in liver glycogen also are graphed for comparison.

tion and the immature liver in the process of normal growth, both seem to know when to stop dividing and when to start dividing again, as if growth processes were integrated with other periodic functions of the organism as a whole.

Acknowledgments

We wish to express our indebtedness to Dr. John J. Bittner for supplying the animals used in these experiments, to Mrs. Olga Hamerston for the glycogen determinations and the mitotic counts, and to Miss Marlys Hawkinson and Mrs. Margaret Scheller for their invaluable help with the many chemical and radio-chemical analyses.

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THE EFFECT OF AZASERINE AND 6-DIAZO-5-OXO-L-NORLEUCINE ON THE BIOSYNTHESIS OF INOSINIC ACID *DE NOVO**

JOHN M. BUCHANAN

Metabolic inhibitors or antimetabolites have frequently played an outstanding role in the unraveling of complex biochemical or biological problems. In turn, a more complete understanding of biochemical reactions has also permitted a more rational approach to the discovery of metabolic inhibitors of some physiological consequence. During the last two years we have had the pleasure of seeing this interaction of physiological and enzymatic experiments in connection with our studies on the biochemical reactions concerned with purine synthesis. The synthesis of purines is an integral part of an even more complex reaction of cells; that is, the synthesis of nucleic acids, compounds which are of vital importance in cellular duplication and growth.

The study of the synthesis of purine compounds was greatly facilitated by the availability of radioactive and stable isotopes for biochemical research. In 1946 a series of compounds labeled with C^{13} was fed to pigeons and an analysis made of the excreted uric acid for the presence within the molecule of heavy carbon. By specific chemical degradations of uric acid it was found that the carboxyl and α-carbon of glycine were the precursors of carbon atoms 4 and 5, respectively, of the purine ring; that formate carbon supplied carbon atoms 2 and 8, and that CO_2 was the precursor of carbon 6. Later experiments, primarily with pigeon liver homogenates, have shown that glycine nitrogen furnishes nitrogen atom 7 of the purine ring; aspartic acid nitrogen, nitrogen atom 1 and the amide nitrogen of glutamine nitrogen, nitrogen atoms 3 and 9 (See Fig. 1).

In addition, G. R. Greenberg of Western Reserve University has shown that the first purine compound formed from these

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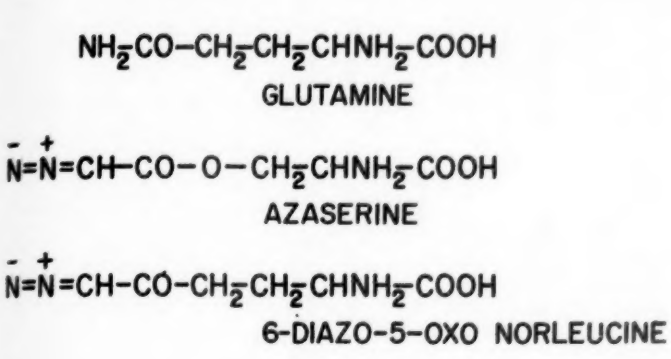

FIG. 1

elementary precursors in pigeon liver homogenates or extracts is inosinic acid. He was also able to show that ribose-5-phosphate when added to pigeon liver homogenates stimulated the synthesis of inosinic acid. From this work it was possible to include ribose-5-phosphate as a precursor of the ribose-phosphate moiety of nucleotides.

The pathway of inosinic acid biosynthesis in pigeon liver described in figure 2 is a result of work done principally in this and Greenberg's laboratory although many important contributions, particularly with microorganisms, have been made by others. In this scheme the precursors of the purines are systematically reacted with succeeding intermediates until the final product, inosinic acid is formed.

In the course of a general program concerned with the effects of antimetabolites on this enzymatic system, we have tested at the suggestion of Dr. Alexander Moore a series of compounds which have been isolated and synthesized as antibiotics by the Parke-Davis Company. Two of these compounds have been O-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (Fig. 3).

Previous experiments by Skipper and his colleagues had shown that the synthesis of nucleic acids in several tissues could be severely depressed by the injection of azaserine in the mouse.

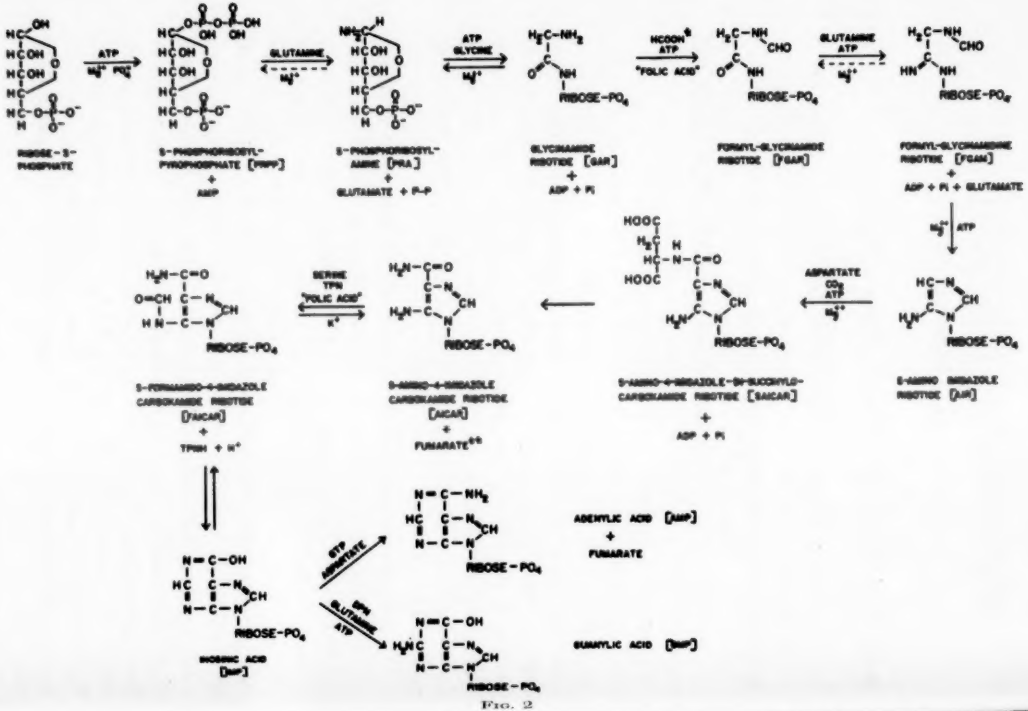
ATP
GLUTAMINE
ATP
GLYCINE
HCOOH
"FOLIC ACID"
GLUTAMINE
ATP
RIBOSE-5-PHOSPHATE
5-PHOSPHORIBOSYL-PYROPHOSPHATE [PRPP]
+ AMP
5-PHOSPHORIBOSYL-AMINE [PRA]
+ GLUTAMATE + P-P
GLYCINAMIDE RIBOTIDE [GAR]
+ ADP + Pi
FORMYL-GLYCINAMIDE RIBOTIDE [FGAR]
FORMYL-GLYCINAMIDINE RIBOTIDE [FGAM]
+ ADP + Pi + GLUTAMATE
ATP
5-AMINO IMIDAZOLE RIBOTIDE [AIR]
ASPARTATE
ATP
5-AMINO-4-IMIDAZOLE-N-SUCCINYLO-CARBOXAMIDE RIBOTIDE [SAICAR]
+ ADP + Pi
SERINE
TPN
"FOLIC ACID"
5-AMINO-4-IMIDAZOLE CARBOXAMIDE RIBOTIDE [AICAR]
+ FUMARATE**
5-FORMAMIDO-4-IMIDAZOLE CARBOXAMIDE RIBOTIDE [FAICAR]
+ TPNH + H+
INOSINIC ACID [IMP]
GTP
ASPARTATE
GTP
GLUTAMINE
ATP
ADENYLIC ACID [AMP]
+ FUMARATE
GUANYLIC ACID [GMP]

FIG. 2

PRECURSORS OF INOSINIC ACID

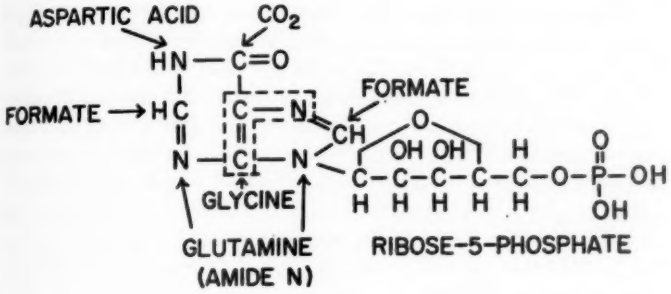

FIG. 3

Upon incubating azaserine with an enzymatic system from pigeon liver it was found that the biosynthesis of inosinic acid was considerably inhibited. However, one of the intermediates of this synthetic pathway accumulated to marked degree. This compound was formylglycinamide ribotide. It was, therefore, believed that azaserine was responsible for blocking the further metabolism of this compound. Upon purification of the various enzymes concerned with inosinic acid biosynthesis it was possible to test the effect of azaserine on each individual reaction.

Azaserine had a mild effect on the reaction:

1.) Glutamine + 5-phosphoribosylpyrophosphate → 5-phosphoribosylamine + glutamic acid + inorganic pyrophosphate

In this reaction azaserine behaved as a competitive inhibitor of glutamine but significant effects of the antibiotic were obtained only at relatively high concentrations of the latter. This, however, was not the case when the effect of azaserine was tested on the reaction.

2.) Glutamine + formylglycinamide ribotide + ATP → formylglycinamidine ribotide + ADP + glutamic acid + inorganic phosphate

Here a pronounced action of azaserine was obtained on the enzyme responsible for the above reaction. Moreover, the degree of inhibition was directly related to the ratio of the concentration

of glutamine to azaserine rather than to the absolute concentrations of either. At a ratio of glutamine to azaserine of 18 the degree of inhibition of the reaction was approximately 50 per cent.

6-Diazo-5-oxo-L-norleucine (DON) was even a more effective inhibitor for this system than was azaserine. When comparisons were made of these two compounds, it was found that at a ratio of glutamine to DON of 727 a 50 per cent inhibition was obtained. This means the DON gives an equivalent response to azaserine at one-fortieth the concentration. It is interesting that this comparison of DON and azaserine in the enzymatic system corresponds very closely to their relative effect in a variety of physiological systems. This would indicate that these two compounds exert their effect *in vivo* by their action on the enzyme system responsible for the conversion of formylglycinamide to formylglycinamidine ribotide.

The action of either azaserine or DON on this enzyme system involves more than a simple competitive relationship with glutamine. In a series of experiments, azaserine was mixed with the enzyme for a brief time (2 minutes) before addition of the other substrates (glutamine, formylglycinamide ribotide and ATP) for the main incubation. With this order of addition of reagents no reaction of formylglycinamide ribotide could be obtained. However, if azaserine and glutamine were added together to the enzyme for the 2-minute interval and then the other substrates added for the main reaction, the expected extent of reaction was observed. Similar experiments have been carried out with DON with identical results. These data indicate that the two antibiotics have a very specific effect on the enzyme system in question. In all probability they compete with glutamine, the natural substrate of the reaction, for the enzyme sites. However, reaction of either compound with the enzyme site probably results in an irreversible denaturation of the enzyme. Thus, glutamine acts competitively with azaserine or DON to delay the inactivation of the enzyme caused by these agents but cannot prevent this inactivation.

The relationship of azaserine and DON to glutamine has more recently been investigated in other systems in other laboratories. Tomisek, Kelly and Skipper have reported that azaserine inhibits the growth of *E. coli* and formylglycinamide ribotide or the riboside accumulates. There is thus no question that these two com-

pounds, azaserine and DON, interfere in the metabolism of glutamine. To date, the main interference is concerned with the conversion of formylglycinamide ribotide to formylglycinamidine ribotide. However, future studies may reveal other equally important sites of its interference with glutamine metabolism.

THYMINE DEFICIENCY AND THE DEATH OF BACTERIA

DR. SEYMOUR S. COHEN*

For the past decade we have been working on bacterial viruses and on the cells which they infect. During the past three years we have come upon some phenomena which seem to be relevant to certain practical aspects of the cancer problem. Some four years ago, Wyatt and I discovered a new pyrimidine, a compound which, until the present time, has been found only in a very few viruses. Indeed this new compound, 5-hydroxymethylcytosine, has not been found in any other cell, or in any nucleic acid and appears uniquely in T-even bacterial viruses. This new substance appears to be at the core of the pathological process in the particular bacterial virus systems with which we have been working.

In attempting to establish the mode of synthesis of the hydroxymethyl compound from pyrimidines such as cytosine, which lack this substitution of position 5, and also in attempting to formulate mechanisms whereby 5-hydroxymethylcytosine or other hydroxymethyl compounds might go to form thymine, namely 5 methyl uracil, a number of metabolic approaches were employed. One of these was to explore the use of certain bacterial mutants requiring pyrimidines for growth. After scouring the country, a single mutant of *E. coli* was obtained which had a thymine requirement. We soon discovered that 5-hydroxymethyluracil did not go to thymine in this organism. Such a transformation would not be expected in any case because of the difficulty of eliminating water from the particular configuration of the hydroxymethyl group at the 5 position. Ordinarily we might have dropped the problem at this point; however, we continued to study this mutant and uncovered a number of rather startling facts which I wish to present for your consideration. These experiments were carried out in collaboration with Miss Hazel Barner.

Initially, it was very difficult to establish a useful relationship

* Department of Biochemistry and Pediatrics, University of Pennsylvania School of Medicine.

between turbidity and viability in cultures of this organism under different conditions of growth. If the organism was suspended in a synthetic medium containing all the requirements for normal growth, namely glucose, nitrogen, phosphorus and mineral salts, in the presence of excess of thymine, the organism multiplied in an apparently normal manner. With limiting thymine, viable count fell off slowly; however, if one omitted thymine entirely the organism did not multiply, stayed at a fixed viable count for approximately 30 minutes and then proceeded to die precipitously, *i.e.*, it lost the power to multiply at the rate of some 90% per division time.

In using the term death, I am defining death as the inability to multiply to form a colony. On a strict application of the definition one may note that for the adult human, this would signify that the brain is dead, an interpretation which is not considered polite, however accurate. Among bacteriologists, such a use of the term is a matter of common practice. One describes ultraviolet irradiation or of various bactericidal agents as killing agents, thereby signifying an action which prevents multiplication or irreversibly destroys the power to multiply, and that is all that "killing" means in this parlance. We shall adopt this terminology for the purposes of this discussion.

Under conditions in which a cell loses the power to multiply, or dies, it is growing. That is so for our thymine-requiring bacterium; it elongates and thickens very considerably, and increases its ribosenucleic acid content, as well as its protein and lipid content. However, the cells do not significantly increase their DNA content, the component characteristic of the nucleus. The cells are growing in a fashion which is unbalanced in contrast to the course of normal bacterial growth and division, in which there is an increase in both cytoplasmic and nuclear substance in a balanced fashion. When the main parts of the cell are out of phase, as they are here, a situation develops which we have termed "unbalanced growth" and which leads to cell death.

I do not wish to imply that the DNA is sitting in a completely inert fashion during this period in which there is no appreciable increase in DNA. It has recently been discovered by Coughlin and Adelberg of the University of California, in working with this mutant and derived mutants that the survivors of thymineless death have undergone a hundred-fold increase in mutation

rate with respect to a variety of inheritable characters. It appears therefore that the chromosomal material is engaged in some type of critical metabolic activity. However, this is not an activity which results in the loss of DNA from the acid precipitable nucleic acids. The DNA appears to be intact with respect to the ability to recover such materials as the phosphorus and thymine which were originally present in the nucleic acid. Nevertheless, if it is imagined, as many believe, that the DNA is the genetic material, we must conclude that the DNA has engaged in activity which has introduced important structural changes.

The cells must synthesize and grow in order to die. We can determine simultaneously the uptake of a carbon source in the absence of thymine, and the loss of viable count. If glucose-grown cells are suspended in glucose it is observed that as the glucose disappears from the medium the viable cells also disappear. If we had grown the cells in glucose and now transfer to xylose during the period in which the cells are unadapted to xylose, the cells do not die. As soon as they have developed the ability to use xylose, the xylose disappears from the medium and the cells die. Using these organisms then, it is possible to show that one may have synthesis of an adaptive enzyme in the absence of DNA synthesis. One might infer that the cytoplasm appears to be the site of synthesis of inducible enzymes.

Now, one would like to be able to extend this phenomenon of killing bacteria to other types of cells. As I have pointed out, this is the only known thymine requiring mutant of *E. coli*. Other cells which have thymine requirements also seem to have multiple requirements, and I interpret this to mean that the presence of an additional requirement in effect preserves the cell against its thymine deficiency. That is, if it has another requirement it will not grow in deficient media and hence, it will not die. Thymine deficiency as a general phenomenon in microorganisms appears to go hand in hand with multiple requirements. Our mutant was picked up quite by accident in a routine screening operation by Dr. Roepke many years ago and the organism has fortunately been preserved.

One might use thymine antagonists to induce a thymine deficiency and 5-bromouracil has been employed with this in mind. Many workers have demonstrated the inhibitory and antithy-

mine properties of bromouracil, and we also undertook to test the compound in some greater detail. I would like to stress that until fairly recently it was customary to explore the efficiency of these inhibitors entirely by turbidimetric methods. I would like to point out that this is a rather dangerous procedure. With our thymine requiring mutant growing in the absence of thymine, we get a turbidometric increase even though the cells are dying. If we give bromouracil, this turbidity increase is very considerable indeed. The turbidity increases at least 6-fold; nevertheless, bromouracil also kills. There is no correlation in this system between viability and turbidity. It may be added that bromouracil stimulates both DNA and RNA synthesis.

Using bromouracil as a thymine replacement, it has been shown by a number of workers that bromouracil will actually be inserted into the DNA of the organism. Most recently, Lipman and Pardee, of the University of California, have demonstrated that if one grows a cell in the presence of bromouracil and infects with bacterial virus, the viruviral progeny contain large numbers of mutants, and appear to contain a great deal of bromouracil in virus DNA. This substance not only may be a thymine replacement and sufficient to produce a pathological DNA, but also may be sufficiently damaging to produce a mutation within a particular organism. I do not know of any studies at the present time which show that bromouracil actually produces a greater mutation rate within bacteria; nevertheless, it apparently does so for viruses.

If cells are given limiting amount of thymine and an excess of bromouracil there appears to be only a slight inhibitory effect if measured turbidimetrically. This is slight only in the sense that there does not appear to be a complete suppression of growth. Indeed, these cells are tremendously long; they are at least 20 times the length of the normal bacteria. From measurements of viable count, it can be seen that a relatively small amount of bromouracil will permit one division and then however, provoke death. Larger amounts produce a tremendous killing effect despite a very slight inhibition of turbidity increase. The killing effect of bromouracil in the presence of thymine may be overcome by thymidine, *i.e.*, thymine deoxyriboside. However, bromouracil desoxyriboside will overcome the protective effect of thymidine.

There is no doubt but that one may provoke the killing of cells having a thymine requirement. However, most strains of *E. coli* do not have a thymine requirement. You cannot insert thymine or thymidine under conditions of normal growth. Using bromouracil we have a situation in which we are replacing thymine by an analogue and are not depriving the cell completely of a pyrimidine which must be inserted into the appropriate position in DNA.

Let us see if we can work out a method of provoking a true thymine deficiency and thereby kill cells which do not normally have a thymine requirement. As you know, sulfanilamide prevents the conversion of PABA to folic acid. Folic acid permits the supply of an essential one carbon fragment to a great many different essential metabolites. These include amino acids such as methionine, serine, histidine and a number of vitamins, *e.g.*, pantothenic acid, thiamine, pyridoxal. Other essential metabolites of this group include the purines, guanine and adenine, and also the pyrimidine, thymine. In other words, a sulfanilamide treated cell is really deficient with respect to all of these metabolites, and it is no surprise then that sulfanilamide provokes bacteriostasis. If a strain of *E. coli* which does not normally require thymine is suspended in the presence of sulfanilamide it can be induced to grow at almost a normal rate by the addition of all of these metabolites. Now some of these metabolites can be eliminated one at a time. If you eliminate any of these except thymine, the cell decreases its rate of growth, but does not die. But, if you specifically eliminate this thymine, supplying the remainder of the essential metabolites, the cells are observed to die very rapidly. The pattern of killing is substantially the same as the pattern of thymineless death which we described previously. In other words we have converted a bacteriostatic substance, sulfanilamide, into a bactericidal substance. Sulfanilamide can be thought of as being bacteriostatic, precisely because it inhibits so many things, so that it inhibits both cytoplasm and nucleus alike. The metabolites, methionine, serine, purine, etc., are important for both cytoplasmic synthesis as well as nuclear synthesis. Advantage can be taken of this by supplying all of the cytoplasmic metabolites; this bacteriostatic material can be converted into an agent which will provoke thymineless death.

The anti-tumor agent, Amethopterin, is a folic acid antagonist and will produce a folic acid deficiency. The recent studies of the mode of action of this substance by Bales and Dancis have indicated that Amethopterin has a much greater effect on DNA synthesis than on RNA synthesis. I think that some of the data which Doctor Skipper presented this morning bear on this point as well. However, this effect does not seem to be absolutely specific. In other words, Amethopterin does seem to have some kind of inhibitory action on RNA synthesis as well as DNA. It may be asked then what would happen if you fed Amethopterin to a tumor-bearing animal, plus all those metabolites essential for RNA synthesis as well as for otherwise normal growth, and specifically omitting from such an enriched diet, thymine or the thymidine precursors. In other words, is it possible that in such a system one might increase the efficacy of this agent in treating tumors? This would be done not by producing a series of inhibitions, if you will, but by increasing the specificity of the inhibition, to provoke what we chose to call "unbalanced growth." I suspect that this kind of a nutritional experiment might be a little difficult to carry out, but I suggest that it might be a rather important one to carry out.

As evidence that an additional deficiency can prevent death, we have prepared a series of double mutants of our thymine requiring organism. In a typical case, we have worked with an organism having thymine plus proline deficiencies. If both materials are given, the organism grows. If thymine is given, omitting proline, no growth is obtained. If we give proline and omit thymine, the usual precipitious killing is obtained; if both are omitted, killing is slowed up enormously and actually the system levels off. Now, if this experiment were analogous to the Amethopterin system you might say that we have a large number of drug-resistant mutants present, *i.e.*, cells which do not die. They are not resistant mutants; they do not die because they are really just slow growers. That is, the protection against killing by thymineless death might be just a function of slow growth, rather than true resistance.

At this point I would like to indulge in some speculation concerning the possible generality of this phenomenon, besides the few systems so far presented. Let us compare a number of bac-

tericidal treatments and see whether they might not fit into this generalized pattern. A fairly representative group would include thymineless death, ultraviolet irradiation, penicillin killing, and nitrogen mustard. Let us look at the effect of the treatment on cell morphology, on DNA synthesis, on RNA synthesis, on the accumulation of various products, and at the requirement for cell growth to produce cell death.

One effect of thymineless death is to produce enlarged or filamentous cells. The treatment inhibits DNA synthesis, maintaining normal RNA synthesis. Uracil accumulates plus a little orotic acid. The cells must grow to die. With UV irradiation the cells very frequently become enlarged. At low doses DNA synthesis is specifically inhibited and RNA synthesis continues. Kanazir and Errera have shown an accumulation of thymidylic acid in such a system. We have shown that ultraviolet irradiated cells must grow to die.

Penicillin killing produces an enlargement of the cells. From our perusal of the literature on penicillin it is quite impossible to make sense of it with respect to an effect on nucleic acid synthesis. I do not know what penicillin does to DNA and RNA synthesis. However, it is well known that nucleotides accumulate. As is also well known, cells must grow in order to die in the presence of penicillin. With nitrogen mustard filamentous and enlarged cells are formed. DNA synthesis is specifically inhibited, while Herriott has shown that RNA synthesis continues. The nature of accumulated products is not known and no one really knows about the relationship of growth to death. Superficially then, one might well imagine that these bactericidal treatments fall into a common pattern, that of inducing death by provoking unbalanced growth. It must be noted that these same types of agents are used rather extensively in tumor treatment. As suggested in our analysis of the Amethopterin data, it can be asked whether the efficacy of all these agents should not be analysed from the point of view of unbalanced growth. In any case it is suggested that any agent which interferes specifically with DNA synthesis and permits normal cytoplasmic synthesis of RNA and protein, is a substance which should produce pathological effects in cells, effects which should kill bacteria and perhaps even tumor cells.

THE EFFECT OF MALIGNANCY UPON NUCLEIC ACID METABOLISM IN NORMAL TISSUES

A. CLARK GRIFFIN*

This presentation will deal with the effects of tumors upon the nucleic acid metabolism and other related functions in normal tissues or organs of the host. Certainly many of these host-tumor interrelations do exist and Stewart (1) has predicted that the eventual control of cancer will come through a better understanding in this area. Greenstein (2) has devoted an entire chapter in his excellent reference text to the tumor-host problem. The best established of the tumor-host effects to date is the depression of the liver catalase that occurs in the presence of cancerous growth. This and many other of the tumor-host relations is adequately discussed by Greenstein.

Cerecedo and associates (3–7) determined the concentration of deoxyribonucleic acids (DNA) and ribonucleic acid (RNA) in many of the organs of normal and tumor bearing mice. In the liver and lungs of the animals with sarcoma, there was an increase in the concentration of both DNA and RNA. These findings were also substantiated by analysis for uracil and thymine. In the kidneys of these tumor bearing animals only the DNA was increased and also the thymine. Regeneration of the liver following partial hepatectomy, however, did not affect the concentration of the DNA or RNA of the normal organs (8).

Kelly and associates (9, 10, 11) have shown that radio-phosphorus is incorporated to a greater extent into the liver, kidney and spleen DNA in mice bearing mammary carcinoma than into the comparable fraction of organs of normal control mice. The effect of the tumor on the incorporation of the labeled phosphate into several normal tissues is shown in Table 1. It may be noted that the DNA of the intestine was not affected by the presence of the tumor.

Since the original observation by Kelly and Jones, others have established that tumors will affect the incorporation of several

* Department of Biochemistry, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

TABLE 1

(From Kelly and Jones Ref. 9)
*Averages of Deoxypentose Nucleic Acid Specific Activities x 10^4**

Tumor wt. per animal	No. of animals	Liver	Spleen	Kidney	Intestine	Tumor
0 (control)	24	.74	36.0	.31	15.1	...
.084 g.	24	2.10	57.8	.93	...	...
1.2 g.	188	3.83	...	..	...	25.0
2.8 g.	24	4.16	67.2	.93	13.0	...

* P^{32} counts/mg. of phosphorus divided by the number of counts injected, normalized for the weight of the mice.

precursors into the DNA fraction of normal tissues. Payne *et al.* (12) reported that C^{14} labeled formate or glycine-2-C^{14} was incorporated to a greater extent into the DNA of several tissues of tumor bearing mice than into comparable fractions obtained from control animals. Tyner *et al.* (13) showed an increase in specific activity of phosphorus^{32} in the deoxyribonucleotides in liver of rats bearing Flexner-Jobling carcinoma compared to control animals at 12 and 48 hours following injection of this isotope. The values in Table 2 are from the findings of Way *et al.* (14). It may be noted that the specific activities of both the adenine and the guanine of the DNA fraction of liver from the tumor bearing mice was several times that of the corresponding components from control animals.

From the above findings we may conclude that the presence of tumors may well affect the nucleic acid metabolism of several tissues of the host. This has been demonstrated in mammary carcinoma in mice, Flexner-Jobling carcinoma in the rat, sarcoma 37, melanoma and lymphosarcoma in the mouse. Also this stimulating effect of the tumor in the DNA of these tissues has been obtained with several nucleic acid precursors: phosphate^{32}, formate-C^{14}, glycine-2-C^{14}, adenine-8-C^{14} and orotic acid-C^{14}.

TABLE 2

(From Way *et al.* Ref. 14)
Specific Activity of Purines in Normal and Tumor Bearing Mice Injected with Adenine-8-C^{14}

	Adenine	Guanine
Control livers	0.23	.03
Livers from tumor bearing animals	0.77	.19
Tumor (Sarcoma-37)	2.84	.71

The problem which now confronts us is one of establishing the mechanism and significance of this effect. Perhaps the solution may be a simple one in that the normal tissues are responding in this manner to the stress placed on the body of the tumor. Certain tissues or organs may be involved in the detoxication and removal of components released by the tumor. Perhaps the tumor requirements for enzymes, metabolic precursor or even preformed macromolecules may be partially met by the host. This would result in increased metabolic demands upon many of the normal tissues. The possibility also exists that the tumor elaborates factors that promote the biosynthesis and mobilization of nucleic acids in some normal organs or tissues. At any rate, a better understanding of this tumor-host relation with respect to nucleic acid metabolism may be of considerable importance. Some investigators have doubted the importance of the several fold change in the incorporation of labeled precursors into the normal tissue DNA that results when a tumor is present in the host. This must be considered as a valid point. However, in most adult tissues the DNA is an extremely stable entity and a several fold increase in the activity of this component fraction, as measured by the incorporation of labeled precursors, would appear to be of significance. In several instances in tumor bearing animals the uptake of labeled precursors into the DNA of normal tissues is increased to the point where it approximates the uptake by the DNA of the tumor.

During the past two years we have further investigated the effect of tumors or of various tumor fractions upon the metabolic activity of the DNA fraction of various normal organs or tissues. C^{57}BL mice bearing mammary carcinoma E-0771 and also normal control mice were injected with adenine-8-C^{14} and were sacrificed three hours or 24 hours later. The DNA fraction was extracted from several organs employing the Schmidt and Thannhauser procedure. Results obtained in this preliminary study are shown in Table 3.

These findings are in essential agreement with those obtained by other investigators. The DNA fraction of the liver, kidney and spleen of the tumor bearing mice incorporated from two to three times more of the adenine-8-C^{14} than the corresponding fraction obtained from organs of normal mice. No essential differences were noted in the uptake of this labeled compound by the DNA from the intestine of normal or tumor bearing mice.

TABLE 3
(Ref. 15)

The Effect of Mammary Carcinoma on the Incorporation of Adenine-8-C^{14} by the Deoxyribonucleic Acid of Mouse Tissues*

Tissue	Time after administration of 2.0 uc. Adenine-8-C^{14} 3 hours	24 hours
Liver (control)	14†	14
Liver (tumor-bearing mice)	12	43
Kidney (control)	10	16
Kidney (tumor-bearing)	8	7
Spleen (control)	605	277
Spleen (tumor-bearing)	1,303	955
Intestine (control)	570	334
Intestine (tumor-bearing)	402	385
Tumor	35	219

* C57BL mice bearing mammary carcinoma E-0771, obtained from Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine.

† Values are reported as disintegrations/second/DNA obtained from 1 gm. fresh tissue. Each value obtained from the pooled tissue of five to seven mice.

The next point to establish was whether the administration of tumor homogenates would alter the DNA metabolism of certain normal tissues as occurs when a tumor is present in the host animal. Injection of fresh homogenates of mammary tumor into normal mice did stimulate the uptake of labeled adenine into the DNA fraction of lung, muscle and thymus. Also, the clear supernatant fraction obtained from the centrifugation of a water homogenate of tumor tissue (2 hours at 20,000 r.p.m., Spinco Model L, 40 head) stimulated up to a three fold intake of adenine-C^{14} into the DNA of the liver, lung and muscle. Several fractionation procedures were applied to the tumor homogenates in order to obtain further information as to the nature of this stimulating factor. From these preliminary studies it was concluded that the factor(s) from the tumor has the following properties (15).

1. It is completely or at least partially inactivated by heat.
2. It is inactivated or solubilized in solutions of low pH.
3. It is rapidly inactivated in tumor homogenates left at room temperature.
4. It is either inactivated or not precipitated by acetone. Further studies now in progress to characterize this DNA stimulatory factor will be considered.

A supplemental study was carried out to ascertain if tumor homogenates or extracts would stimulate adenine uptake into

mouse lung and liver *in vitro* (16). The livers and lungs of young C57BL mice were minced and supplemented with Ringer's solution fortified with glucose and phosphate buffer. Adenine-8-C^{14} was added and tumor homogenate was added and the tubes were allowed to incubate for four hours. Following this incubation period the DNA was isolated from the mixture by the procedure of Schmidt and Thannhauser. The values of radioactivity for the DNA fractions are shown in Table 4.

TABLE 4
(Ref. 16)

Values of Radioactivity for DNA Extracted After Incubation with Adenine-8-C^{14}

Group	Treatment of Tissue Mince	Dis./sec./g wet wt. of washed mince tissue
1.	Untreated	4.8, 7.5
2.	Tissue mince heated 5 min. in boiling water	.9, 1.2
3.	Tissue mince + 100–200 mg. homogenized tumor tissue	8.3
4.	Tissue mince + lyophilized tumor homogenate corresponding to 400 mg. tumor tissue	6.0, 8.9
5.	Untreated	5.5
6.	Tissue mince + 25 mg. tumor homogenate	18.4
7.	Tissue mince + 50 mg. tumor homogenate	16.2

Again these preliminary *in vitro* studies indicate that tumor tissues are capable of stimulating the incorporation of adenine into the DNA of certain normal organs. The liver DNA fraction proved to be more responsive than the DNA fraction of lung.

We hope eventually to isolate and further purify the factor(s) presumably present in the tumor tissues that is responsible for the above noted DNA stimulatory effect. Several studies are in progress in order to establish a test system that will provide the maximum in response and in sensitivity for the testing which must accompany the fractionation of the tumor. In this survey program it has been noted that several tumors are capable of stimulating the uptake of adenine-8-C^{14} into the DNA fraction of muscle tissue closely adjacent to the tumor. Some of these findings are illustrated in Table 5 (S. M. Grundy and A. C. Griffin, unpublished data).

From these results it may be observed that the mammary carcinoma and melanoma and lymphosarcoma to a lesser extent do exert considerable effect on the DNA metabolism of adjacent muscle

tissues. This increased activity of adjacent muscle may be due to some nonspecific localized tissue reactions. However, some evidence has been obtained that does not support this contention. Whether or not this DNA activity of the muscle tissue adjacent to tumor may be the result of some specific factor elaborated by the tumor must await further investigation.

TABLE 5

The Effect of Mammary Carcinoma, Cloudman Melanoma, or Gardner Lymphosarcoma On the Incorporation of Adenine-8-C^{14} by the Deoxyribonucleic Acid of Mouse Muscle

	Tissue	Counts in DNA	
Mammary Carcinoma			
Run I	Tumor	29.5	(A)
	Adjacent Muscle (tumor-bearing)	33.6	
	Opposite Muscle (tumor-bearing)	2.3	
	Control Muscle (normal)	0.3	
Run II	Tumor	36.0	(A)
	Adjacent Muscle (tumor-bearing)	28.0	
	Opposite Muscle (tumor-bearing)	0.3	
	Control Muscle (normal)	4.0	
Cloudman Melanoma			
Run I	Tumor	65.1	(A)
	Adjacent Muscle (tumor-bearing)	8.4	
	Opposite Muscle (tumor-bearing)	0.3	
	Control Muscle (normal)	0.0	
Run II	Tumor	40.4	(A)
	Adjacent Muscle (tumor-bearing)	15.1	
	Opposite Muscle (tumor-bearing)	0.0	
	Control Muscle (normal)	0.9	
Gardner Lymphosarcoma			
	Tumor (rapid growing)	83.0	(B)
	Adjacent Muscle (rapid growing tumor)	15.0	
	Adjacent Muscle (slow growing tumor)	3.1	

A—DNA isolated by the procedure of Schmidt and Thannhauser.
B — DNA isolated by the salt extraction procedure of Barnes and Schoenheimer.

Other workers have obtained evidence that the presence of a tumor may alter the nucleic acid metabolism of the normal tissues of the host. Only a few of these studies will be cited. Anderson *et al.* (17) have reported that the turnover of both DNA and RNA was higher in livers of tumor bearing mice than in that of normal animals. There is an increased incorporation of hypoxanthine into the RNA adenine of the tissues of tumor bearing hamsters according to Balis *et al.* (18). Annau *et al.* (19) and Malmgren

(20) have reported an increased mitotic rate in the livers of tumor bearing mice and rats.

Observations from this and other laboratories suggest that cancerous growths may have considerable impact on the composition and metabolism of the normal tissues of the body. There is not sufficient evidence as yet to assign any definite significance to this tumor-host relation. Indeed, many of the effects noted may be only of secondary importance and reflect the body's normal response to the invasion by the malignant tissues. The extent to which the tumor may utilize various metabolites synthesized or preformed by the host has not been fully established. Of interest in this respect is the relatively high utilization of serum proteins and other macromolecular structures by tumors (Babson and Winnick 21-22 and Busch and Greene 23). Finally, the establishment of the presence of factors in malignant or other rapidly growing tissues capable of stimulating the synthesis or the mobilization of nucleic acids would appear most worthwhile. A further elucidation of the mechanism of nucleic acids or nucleoprotein formation, associated with cellular division is required before we may differentiate normal from abnormal growth processes. Recently, Okumara *et al.* (24) have reported that relatively simple structures such as kinetin (6-furfurylaminopurine) obtained from heated DNA are capable of stimulating cell division in tobacco pith segments. It will be of interest to determine if there are substances present in tumors, whether of a simple nature as the kinetins, or of a more complex nature, which are involved in the stimulation of nucleic acid metabolism.

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PURINE SYNTHESIS IN ASCITES TUMOR CELLS*

G. A. LePage and Alan C. Sartorelli

We wish to take advantage of the chairman's invitation to express some of our research philosophy by commenting on our views of cancer chemotherapy and their relation to the project on which we are reporting. These views are by no means represented as original, and have no doubt occurred to others working in this field.

(a) It appears probable that in the next few years we shall acquire a series of chemical agents that are effective as inhibitors of cancer.

(b) Cancer appears to be not one disease but a variety of diseases having only a few characteristics in common. An analogy with infectious diseases might be made. Each infectious disease involves a different organism, has certain symptoms in common with other infections, varies in some of the effects on the host and in the treatment to which it responds.

(c) Very much as we have had a series of antibiotics developed, each effective against some infectious agents and not others, the anticancer drugs will probably each affect only a small percentage of a spectrum of cancers.

(d) In order to operate an effective cancer chemotherapy, it will be necessary to work out the mechanism of action of each anticancer drug, study the mechanisms of resistance to it, and have biological test systems to determine which drugs to use in a specific case.

We have been studying purine biosynthesis. The results we are reporting represent the first phase of an attempt to apply the aforementioned philosophy in terms of agents active on this section of cell metabolism.

To date we know of two metabolic pathways by which cells can make nucleic acid purine nucleotides. These are represented

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in the first chart. One is termed "de novo" synthesis—the purine structure is built up from small precursors such as formate, glycine and carbon dioxide. The other involves utilization of preformed purines from the diet or from other cells. We measure these synthetic pathways with radiocarbon-labeled precursors, glycine-2-C^{14} for de novo synthesis and adenine-8-C^{14} or its nucleotide derivative for the utilization of preformed purines. We have used ascites cell tumors (tumors growing in the peritoneal cavity in accumulated fluid as suspensions of free cells) in two general test systems. In one the precursors and test compounds are injected intraperitoneally in ascites cell tumor bearing mice. This provides in vivo test on the action of tumor cells, relatively independent of the usually superimposed factors of circulation and excretion. We effectively obtain an intratumoral injection. The second test system uses washed ascites cells, in vitro, in Warburg respirometer vessels of 60 ml. capacity, with 150 mg. ascites cells in 12 ml. of salts-glucose medium at 38° C under 95% N_2-5% CO_2. This permits use of a more controlled environment. In both cases we use ion-exchange and paper chromatography to isolate acid-soluble purine compounds and the nucleic acid purines, adenine and guanine (Le Page, 1953). Where it is necessary, we separate ribonucleic acids from deoxyribonucleic acids. But routinely in the work reported here, they were treated as a pool and extracted and hydrolyzed to free purine bases together by means of hot acid. The ascites cell tumors incorporate adenine-8-C^{14} and glycine-2-C^{14} into both RNA and DNA, with approximately twice as much going to RNA as DNA in short incubations. The rates of incorporation for the in vivo and in vitro systems are roughly comparable.

Four ascites cell tumors were compared for their relative abilities to incorporate adenine-8-C^{14} and glycine-2-C^{14} in vivo. The results are shown in Tables 1, 2. Some of these data have been reported elsewhere (Fernandes, *et al.*, 1956; Le Page, *et al.*, 1955). It is evident that the four tumors differ widely in the relative balances of the two purine nucleotide synthesis systems. Ehrlich carcinoma cells have the lowest rate of adenine utilization of the four tumors. Ehrlich cells are more efficient at de novo purine synthesis than is indicated in Table 1. Determination of the specific radioactivity of the glycine extracted from the cells after a 1-hour incubation showed that the endogenous glycine

had diluted the glycine-2-C^{14} twice as much in Ehrlich cells as in the other three tumor cell types.

One of the most interesting model compounds we have had for the study of the premises outlined earlier, is azaserine, a compound now being used in the treatment of acute leukemias. It is a very potent inhibitor of de novo purine synthesis. The groups

TABLE 1

Rate of incorporation of glycine-2-C^{14} into ascites tumor cell purines in vivo. *25–28 gm. mice were each given 100 microgms. glycine-2-C^{14} (1.8×10^6 c/min.) I.P. and cells inactivated after 1 hr.*

Tumor	NA adenine Cts./min./microm.	NA guanine Cts./min./microm.
Ehrlich	39 ± 4*	60 ± 5*
Sarcoma 180	73 ± 11	44 ± 14
TA3	248 ± 48	190 ± 14
6C3HED	33 ± 11	20 ± 6

Each figure is an average of analyses on 3–9 mice.

* These figures would be approximately twofold higher if correction were made for net specific activity of glycine-2-C^{14} in the cells. Ehrlich cells appear to have higher endogenous glycine pools and the precursor is diluted

Survival time given in days. Bracketed figure is the no. of mice.

TABLE 2

In vivo *rate of incorporation of Adenine-8-C^{14} by Ascites Tumor Cells. 25–28 gm. mice were each injected I.P. with 20 microgms. adenine-8-C^{14} (97000 c/min.) and inactivated after 1 hr.*

Tumor	NA adenine Cts./min./microm.	NA guanine Cts./min./microm.
Ehrlich	67 ± 2.4	5.3 ± 0.5
Sarcoma 180	71 ± 4	5.4 ± 0.4
TA3	131 ± 5	5.6 ± 0.7
6C3HED	273 ± 19	9.1 ± 0.6

Each figure is an average of analyses of from 5–15 mice.

in the laboratories of Dr. Skipper (Skipper, *et al.*, 1954) and Dr. Buchanan (Hartman, *et al.*, 1954; Levenberg, *et al.*, 1956) have studied the action of azaserine in E. coli and pigeon liver systems respectively, and shown that its effects are such that it prevents the purine precursor N-formyl glycinamide ribotide from being transformed into N-formyl glycinamidine ribotide by reaction with glutamine. It is thus referred to as a glutamine antagonist.

We have found azaserine to be a very effective inhibitor of de

novo purine synthesis in all four of the ascites cell tumors studied (Fernandes, *et al.*, 1956; Le Page and Greenlees, 1955). The intraperitoneal injection of 5 microgm. or less into an ascites tumor bearing mouse gives essentially complete inhibition of glycine-2-C^{14} incorporation in the purines for approximately 12 hours. The addition of 0.1 microgm. of azaserine to a 12 ml. reaction mixture containing 150 mg. wet weight of ascites cells in vitro gives 90–97% inhibition. The 5 microgm. per 25 gm. mouse in vivo dose does not affect de novo synthesis in the spleen, but when the dose is raised some 5 fold, the spleen is also inhibited to this extent. This may not seem a valid comparison until it is considered that the azaserine takes 5–15 minutes to act on the ascites cells, that the glycine must travel the same route from peritoneum to spleen in the test, and that 2, 4 diamino-5-(3′, 4′-dichlorophenyl) 6-ethylpyrimidine, another inhibitor, affects the spleen more than the tumor when administered by the same route.

Experiments were set up to test the effects of azaserine on the survival of mice bearing ascites cell tumors, bearing in mind the observed duration of the biochemical effect. Control groups were injected I.P. with isotonic saline. One group was injected once a day I.P. with saline containing 10 microgm. azaserine. A third group received 10 microgm. per mouse per day in two equal doses approximately equally spaced (*i.e.* 12 hrs. apart). Treatment was started 24 hours after the tumors were transplanted and continued for 6 days only. Then survival times were observed. The results are shown in Table 3. The correlation between duration of the biochemical effect and prolongation of survival is quite apparent.

Azaserine is quite water soluble. From the 12 hour duration

CHART I - OUTLINE OF RESEARCH

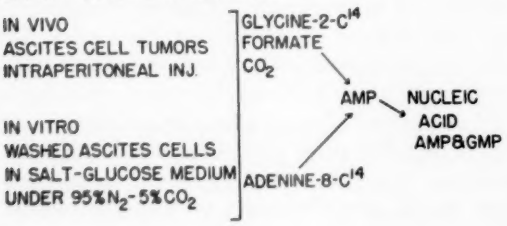

TABLE 3

Effect of azaserine on survival time of Ascites Tumor Bearing Mice.

Tumor	Mice	Controls	Azaserine 1 x daily	2 x daily
			days	
Ehrlich	Swiss	15(16)	18(8)	21(18)
TA3	CAF1	17(23)	18(13)	25(18)
Sarcoma 180	Swiss	8(10)	13(10)	18(8)*
6C3HED	C3H	13(10)	17(10)	22(10)

Survival time given in days. Bracketed figure is the number of mice.

* Two others survived without evidence of tumors.

of its effects, we suspected that some binding to cell constituents might be involved. That such is the case is indicated by several different tests. Mice bearing Ehrlich ascites cell tumors were relieved of approximately half the tumor cell suspension by an abdominal puncture and each injected I.P. with 5 microgms. of azaserine. The remaining cells were withdrawn 1 hour later. Both batches of cells were washed by repeatedly suspending them in isotonic saline and centrifuging. This is a procedure which efficiently removes endogenous glycine. When the two batches of cells were compared for ability to incorporate glycine-2-C^{14} in

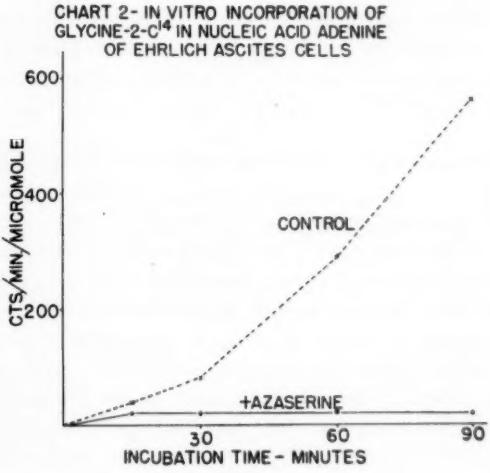

CHART 2

vitro, the results shown in Table 4 were obtained. Azaserine inhibition was not relieved by washing the cells.

Chart 2 illustrates a time curve for glycine-2-C^{14} incorporation in vitro in control flasks and flasks treated with 0.5 microgm. azaserine. Each flask had 150 mg. Ehrlich cells. It appears that azaserine takes approximately 10 minutes to combine with a cell constituent, after which the de novo synthesis is completely blocked.

TABLE 4

Effect of pretreatment with azaserine in vivo *on incorporation of glycine-2-C^{14}* in vitro

Tissue	Acid-sol. adenine cts./min./micromole	NA adenine
Control cells withdrawn before azaserine treatment	5800	270
Control cells + 0.5 microgm. azaserine added *in vitro*	78	0
Cells from azaserine treated mice washed repeatedly with saline	108	0

Liver is known to contain an enzyme which deaminates and thereby inactivates azaserine (Reilly, 1955). Addition of mouse liver homogenate (30 mg.) to flasks containing 150 mg. of Ehrlich cells incubated in vitro had no influence on the inhibitory effect of azaserine added 15 minutes earlier, but when added at the same time as the azaserine it partially relieved inhibition. Azaserine added before the liver enzyme was therefore unavailable to it.

Experiments were conducted with two ascites cell tumors (TA3, Ehrlich) in an attempt to determine whether there was an azaserine-resistant component in their cell populations. Mice were given intraperitoneal transplants with 10^7 cells each. Each mouse was given an I.P. dose of azaserine 1, 2, 3 and 4 days after the transplant. On the seventh day, cells were withdrawn and used to transplant again. At 7 days there were approximately half as many cells as in untreated controls, 4–5 x 10^9 cells. This regimen was continued for 19 transfers in the Ehrlich, for 18 in TA3, with the azaserine dose increased 50% at the fifteenth transfer. Tests for susceptibility to azaserine were conducted at intervals. Data for some tests on Ehrlich cells are reported in

TABLE 5

Tests for susceptibility of Ehrlich ascites cells to azaserine with continued treatment. Transplants with 10^7 cells were made every 7 days, azaserine treatment on days 1, 2, 3, 4 after transplanting, with 0.2 mg./kg. for 15 transfer generations, then 0.3 mg./kg. Test mice used on 6th day were given azaserine. Three hrs. later a dose of 100 microgms. glycine-2-C^{14}, incubated 1 hr. Figures are averages of analyses from 4 mice.

Generation	Azaserine mg./kg.	Acid-sol. adenine cts./min./microm.	NA adenine cts./min./microm.
Control	0	1208 ± 110	115 ± 15
3	0.04	395 ± 45	48 ± 3
3	0.2	28 ± 11	9.1 ± 2
6	0.04	55 ± 9	3.3 ± 0.2
6	0.2	50 ± 11	7.0 ± 2.5
19	0.04	16 ± 3	0.4 ± 0.2
19	0.2	20 ± 3	0.9 ± 0.3

TABLE 6

Tests on adenine-8-C^{14} incorporation in azaserine treated ascites tumor cells. Mice given I.P. transplants of TA3 cells were treated with azaserine (0.2 mg./kg.) on days 1, 2, 3, 4 and used for tests on adenine incorporation on day 6. Mice received 25 microgms. adenine-8-C^{14}, I.P., and were sacrificed after 1 hr. Each figure is on average of analyses from 4 mice.

Generation	NA adenine cts./min./microm.	Acid-sol. adenine cts./min./microm.
Control	64.3 ± 0.7	930 ± 32
1	154 ± 9.6	1890 ± 81
Control	45.7 ± 10	615 ± 170
12	95.3 ± 8.9	1063 ± 110
Control	78.4 ± 13	874 ± 390
18	74.4 ± 15	907 ± 146

Table 5. No significant selection appears to have occurred, at least in terms of susceptibility of de novo purine synthesis.

We were also interested in determining what happened to the ability of the cells to use preformed purines during this attempted selection. Table 6 shows results of such tests. The radioactivity from adenine-8-C^{14} reaching nucleic acid adenine increases over control at the first transfer generation, but recedes to control or lower by the eighteenth generation. However, the specific activities of the acid-soluble adenines vary widely. Table 7 shows a recalculation of the nucleic acid adenine figures with correction to equal specific activities for the acid-soluble adenine

TABLE 7

Adenine-8-C^{14} incorporation of azaserine treated TA3 cells in vivo—corrected to constant specific activity of acid-soluble adenine.

(See Table 6).

Generation	NA adenine
Control	100
1	118
12	121
18	92

(assumed to contain the precusor). From this it is evident that adenine utilization for nucleic acid synthesis did not change very much with azaserine treatment. But the size of the acid-soluble nucleotide pool must have decreased to less than half of control with the first series of azaserine treatments. After 18 transfers it had returned to control levels.

Dr. Buchanan's group have reported (Hartman, *et al.*, 1954) that the addition of azaserine to preparations of pigeon liver which are synthesizing inosinic acid leads to the accumulation of relatively large amounts of two intermediates, glycinamide ribotide (GAR) and ∝-N formyl glycinamide ribotide (FGAR). We were interested in determining whether this occurred in the ascites tumor cells. An ion-exchange chromatography system was set up for the separation of these compounds from perchloric acid extracts of tissues. Chart 3 shows their location on gradient elution chromatograms from Dowex-1 formate columns eluted with formic acid in 5 ml. fractions. The position of FGAR was established with an authentic sample.[1] The acid-soluble extracts from flasks incubated 1 hour in vitro with 150 mg. wet weight of Ehrlich cells, 150 microgms. glycine-2-C^{14} and 0.5 microgms. azaserine were chromatographed under the conditions used to obtain Chart 3. No peak of radioactivity was found where FGAR should appear. A peak of radioactivity did appear, for certain conditions, where GAR is indicated on Chart 3. This material has been further purified and analyzed for ribose, phosphorus, nitrogen and ninhydrin reaction. The analyses agree well with those for GAR. Table 8 shows some data obtained for the in vitro

[1] We are grateful to Dr. G. Robert Greenberg for a gift of some radiocarbon labeled N-formyl glycinamide ribotide.

TABLE 8

Accumulations of radioactivity as GAR in Ehrlich ascites cells (150 mg.) incubated with glycine-2-C^{14} (1.86 x 10^6 c/min./microm.), azaserine (0.5 microgms.) and glutamine (1 microm.).

Additions	GAR c/min.	Acid-sol. adenine c/min./micromole
0	0	17500
Formate + ribose-P + ammonia	0	17900
Glutamine	16,900	31000
Azaserine	0	130
Azaserine + glutamine	64,800	8000

incubations. The highest accumulation shown represents only 0.04 micromoles of GAR.

Ehrlich cells incubated for 1 hour in vivo with glycine-2-C^{14} were extracted and the extracts chromatographed. No radioactivity appeared in the FGAR or GAR regions. But when azaserine was injected, the cells accumulated relatively large amounts of GAR. No FGAR peak could be detected. Table 9 shows some of the data from these tests. In azaserine-treated cells, 4.7% of the injected radioactivity appeared in the GAR peak after a 1 hour incubation. The amount of GAR accumulated reached, on a molar basis, the same level as the sum of the AMP-ADP-ATP present in the cells. The administration of a large dose of glutamine (20 micromoles per mouse) simultaneously with the azaserine and glycine-2-C^{14} considerably depressed the GAR accumulation and largely relieved the inhibition of incorporation into adenine. Delay in injecting the glycine-2-C^{14} for fifteen minutes largely abolished the protective effect of glutamine on in-

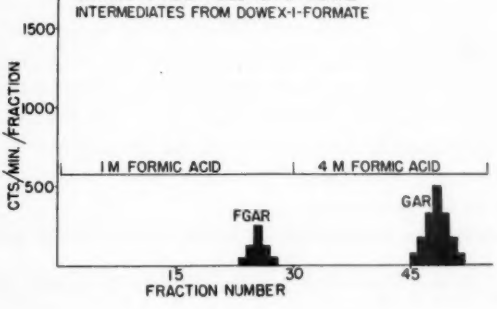

TABLE 9

In vivo experiments on GAR accumulation with mice bearing Ehrlich ascites cell tumors. Each treated mouse received 100 microgms. glycine-2-C^{14} (2.48×10^6 c/min.) and 5 microgms. azaserine. Incubation with glycine-2-C^{14} was 1 hr. Five mice were used for each figure.

Treatment	GAR c/min./ml. cells	Acid-sol. adenine c/min./micromole
Control	0	8100
+ Azaserine	154,000	50
+ Azaserine + 20 microm. glutamine simultaneously	28,300	6900
+ Azaserine + 20 microm. glutamine with the glycine-2-C^{14} given 15 min. after glutamine and azaserine	120,000	2100
+ Azaserine + 40 mg./kg. of methionine sulfoximine	66,200	43

corporation into adenine and restored accumulation in GAR to 78% of that obtained without glutamine. Either the glutamine is destroyed very rapidly or glutamine cannot reverse the effects of azaserine once it is bound to the cells. Support for the latter was obtained with the in vitro system, where it could be shown that the glutamine was not destroyed. Flasks containing glutamine and azaserine show incorporations in adenine that are 35% of control. When the cells are incubated 15 minutes with azaserine before glutamine and glycine-2-C^{14} are added, incorporation is only 3% of control. Thus glutamine could partly prevent azaserine inhibition but not reverse it.

Methionine sulfoximine,[2] which has been reported as an inhibitor of glutamine synthesis (Pace and McDermott, 1952), reduces in vivo accumulation of radioactivity in GAR by Ehrlich cells to 43% of that obtained with azaserine alone. In TA3 cells it has no such effect. Combination treatment of mice bearing these tumors indicates that survival time is greater in groups receiving azaserine only than in those receiving azaserine plus methionine sulfoximine. However, this was equally true of the tumor susceptible to methionine sulfoximine (Erhlich) and the resistant one (TA3). Hence the effect must relate to action of the drug on the host tissues.

We have then, experimental tumors and a compound which

[2] We are grateful to Dr. Karl Folkers of Merck and Co. for a gift of some methionine sulfoximine.

inhibits their de novo purine synthesis essentially 100%. These tumors have been demonstrated to use both de novo synthesis and preformed purine utilization to make their nucleic acid purine nucleotides. If an inhibitor of preformed purine utilization were available, a test could be made as to whether our biochemical tests permit prediction of the success of these agents against a given tumor. We found that the chloramphenicol isomer 1(+) threo chloramphenicol,[3] which is not active as an antibiotic, inhibited the in vitro incorporation of radiocarbon-labeled adenosine-5-phosphate into ascites cell nucleic acids. The incorporation of adenine-8-C^{14} on the other hand was not inhibited in either in vitro or in vivo systems. Table 10 presents data from these experiments. Survival tests were set up with groups of mice bearing TA3 transplants. The results are shown in Table 11. The mice were treated for 6 days. The observed inhibition of the in vitro utilization of AMP-C^{14} was 57%. Increase in survival time was 4 days or 66% of the treatment time. Thus the observed inhibition of AMP utilization correlates with in vivo effect on survival. The indication is that for a tumor possessing ability to use both pathways for nucleic acid purine synthesis, a successful

TABLE 10

Incorporation of AMP-C^{14} and adenine-8-C^{14} into TA3 ascites cells in vitro.

Additions	NA adenine c/min./micromole
AMP-C^{14}	275
AMP-C^{14} + chloramphenicol	119
Adenine-8-C^{14}	19,900
Adenine-8-C^{14} + chloramphenicol	20,700

TABLE 11

Chemotherapy tests with azaserine and chloramphenicol using groups of 8 mice bearing TA3 ascites tumors treated twice daily for 6 days, starting 1 day after the tumor transplanting.

Treatment	Survival days
Saline	17
Azaserine, 0.2 mg./kg./dose	25
Azaserine + chloramphenicol at 50 mg./kg./dose	29

[3] We are grateful to Major Charles L. Wisseman, Jr., and to Parke-Davis and Co. for gifts of the 1(+) threo chloramphenicol, and to the latter for a gift of azaserine.

therapy could be expected only with an agent or agents capable of inhibiting both pathways completely. The absence of any inhibitory effect by chloramphenicol on the incorporation of adenine-8-C^{14} may mean that there are alternate pathways for adenine to reach nucleic acids other than by way of AMP. However, it seems more reasonable to interpret this result in terms of permeability and compartmentation of reactions within cell particulates. This last experiment illustrates the necessity for detailed knowledge of the metabolism in devising biochemical tests such as were proposed in the introduction.

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THE DISTRIBUTION OF NEWLY SYNTHESIZED DNA IN MITOTIC DIVISION

WALTER PLAUT* AND DANIEL MAZIA†

One of the more intriguing processes which take place within a cell in the course of its preparation for division is the duplication of the chromosomes. The investigative methods of classical cytology and genetics have pointed to some of the conditions which must be satisfied by the mechanism responsible for this process. These methods have not, however, given us much insight into the nature of the mechanism itself. It has been shown that chromosomal duplication involves the production of two functionally and structurally alike chromosomes from one. Speculation on the nature of this process has been hindered by the absence of answers to basic questions on the process. Perhaps the most fundamental of these questions is whether, in the course of reproduction, the material of the old chromosome is partitioned equally between the two daughter units or whether the old chromosome persists and determines the formation of an essentially new unit. It is this question to which we have attempted an experimental approach.

Experimentally the question becomes one of distinguishing between newly synthesized and old chromosomal material. If we use isotopic labeling to make this distinction and introduce our markers during the period of chromosomal reproduction, the products of this reproduction should then permit us to distinguish between the two alternatives to be tested. If the material of the parent chromosome is equally divided between the daughters, each of these should have the same amount of newly synthesized, marked material. If, on the other hand, reproduction involves the new synthesis of an entire unit, the daughter chromosomes should be unequally marked. The experiment must be designed, then, to provide a maximal chance of detecting such inequality, should it occur.

In translating this experimental approach into practicability,

* Botany Department, University of Wisconsin, Madison, Wisconsin.
† Zoology Department, University of California, Berkeley, California.

the ideal of labeling all of the chromosomal material during the duplication of one chromosome and then analyzing the distribution of the label between the two resulting daughter chromosomes had to be abandoned in favor of a more practical design. DNA was chosen as the chromosomal constituent to be labeled. This molecule was selected because it is a constant structural component of the chromosome and is not found in non-chromosomal cell structures. By restricting the analysis to one molecular constituent we will obviously know the chromosome only by that constituent, the DNA molecule. On the other hand, we may gain some information on the behavior of that molecule, which is of interest in itself. Instead of dealing with a single chromosome and the products of its reproduction, we chose a plant cell with a low number of chromosomes, which permitted clear cytological observation during the mitotic stages following chromosome duplication. Obviously, if the parental chromosome divides its material equally between two daughters, then the number of such sets of daughters will have no effect on the expected result: new material will be equally distributed between the two sets of daughters constituting the two new nuclei. A large number of such sets could, however obscure an unequal distribution of new material between individual daughter sets since the orientation of these daughters at metaphase is presumably random. The plant selected, *Crepis capillaris* has a somatic chromosome number of six. We felt this number to be low enough to permit the detection of possible inequality.

The essence of the experiment is illustrated in figure 1, which represents the pertinent events during one mitotic cycle. There is a period of DNA synthesis in the interphase of the cycle. It is during this period that chromosomal duplication must occur. This, then, is the time when the new chromosomal material must be marked. The results of chromosomal reproduction can be studied in the ensuing anaphase or telophase, when the products of chromosomal duplication have become separated in their movement to opposite poles of the dividing cell. If one exposes a population of cells to the labeled DNA precursor for a period sufficiently long for a cell to proceed from interphase synthesis to anaphase or telophase and fixes them, then the radioactivity in the whole of an anaphase or telophase cell is a measure of DNA

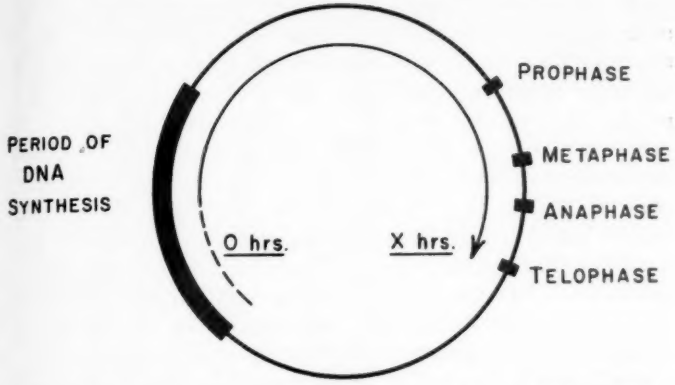

FIG. 1. Schematic representation of mitotic cycle with period of DNA synthesis placed in relation to morphologically recognizable stages. (Not drawn to scale.)

synthesis in the preceding interphase, and the distribution of radioactivity between the two halves of such a cell represents the mitotic distribution of this newly synthesized chromosomal material.

The remaining practical problems are those of the detection and measurement of the radioactivity and the isotopic label to be employed. Since detection must be carried out *in situ*, where the radioactivity can be correlated with cytological detail, *i.e.*, the chromosome groups, the use of an autoradiographic method was clearly indicated. We chose stripping film autoradiography (Doniach and Pelc, 1950) for its high resolution potential and its adaptability to instrumental quantitation (Mazia *et al.*, 1955). Thymidine-2-C^{14} was selected as the radioactive precursor for DNA since it had been demonstrated to be incorporated only into the DNA fraction of the cell (McQuade *et al.*, 1955).

Seeds of *Crepis capillaris* were germinated on filter paper. The resultant seedlings were transferred to a culture solution for incorporation of the isotopic marker. This solution consisted of glass-distilled water and C^{14}-thymidine at a level of 0.5 to 0.8 μC per milliliter. After 12 hours of growth in this medium the seedlings were fixed in acetic alcohol, macerated in N HCl for three to four minutes at 60° C, and their root tips squashed on slides. The growth period of 12 hours was found to be enough

for a cell to proceed from interphase synthesis to telophase. (In other words, the value for X in figure 1 is 12 hours or less.) The slides were then coated with autoradiographic stripping film and stored for exposure periods of six and twelve days. After photographic development the slides were subjected to analysis.

Qualitative observation of the autoradiographs showed that the recorded radioactivity of an appreciable fraction of the anaphase and telophase cells was not distributed equally between the two halves of the dividing cell, suggesting that new DNA was not distributed equally between the products of chromosomal reproduction (see Plate I). In order to permit a more objective evaluation of this distribution, the preparations were subjected to an instrumental quantitation of the autoradiographs.

Cytologically clear cells were photographed twice, at the same magnification: first with phase contrast illumination to record the cytological detail, then in brightfield, to show the distribution of silver grains in the autoradiographic emulsion over the cells. (For details on optical arrangements and photomicrography see Plaut and Mazia, 1956.) By superimposing the two negatives of any cell, the areas of the brightfield negative corresponding to chromosomal groups could be objectively marked. This marking process is illustrated in figure 2. The marked brightfield negative was then analyzed by the densitometric instrument outlined in figure 3. A sample record of such an analysis is shown in figure 4: the peaks in the curve represent markings made in the brightfield negative to indicate the physical extent of chromosome groups, the smooth curve sections between these peaks correspond to the light transmitted by the images of grains over chromosomes. The areas under these smooth curve sections are thus a measure of the number of grains, *i.e.*, the amount of radioactivity within each of the two halves of a dividing cell in anaphase or

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Figures 1a and 2a are phase contrast photomicrographs, 1b and 2b brightfield photomicrographs of the corresponding regions in the autoradiographic emulsion. Magnification is about 1600 X for 1a and 1b, about 2500 x for 2a and 2b. Quantitation of these cells gave the following results:

Telophase in figure 1: 238 and 96 (in arbitrary units), a ratio of 2.5:1.0.

Prophase in figure 1: 343. (Note the good agreement between the summed division products, 238 + 96, and the pre-division, but post-synthesis chromosomes of the prophase.)

Telophase in figure 2: 93 and 87, a ratio of 1.1:1.0.

The spatial correspondence of grain clusters and chromosome groups should be noted. In addition to these dense grain patterns which are produced by the C^{14} beta particles emanating from labeled DNA there are also present more or less uniformly distributed background grains.

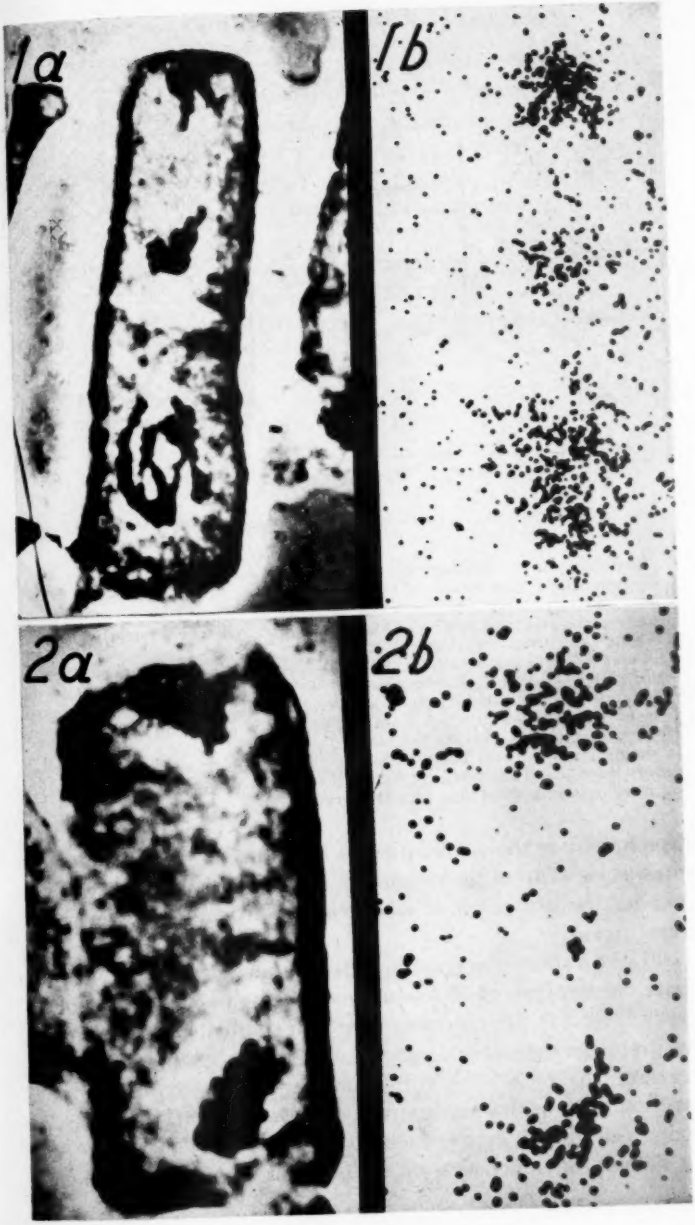

PLATE I

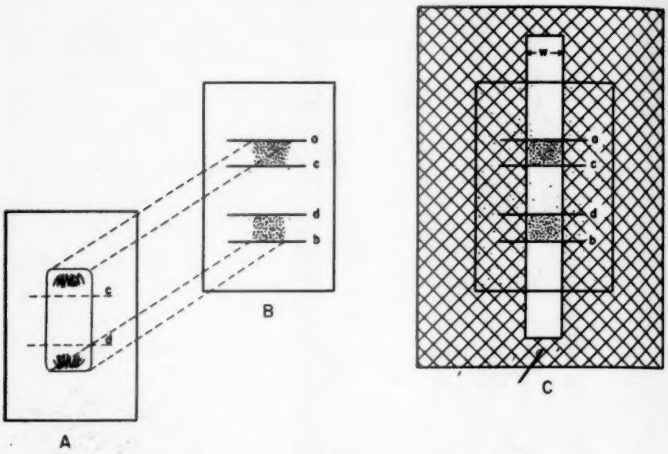

FIG. 2. Diagrammatic illustration of the preparation of a brightfield negative for scanning.

A—phase contrast photograph of a telophase.
B—brightfield photograph of the autoradiograph of the telophase.
Since the two photographs are taken at the same magnification, they can be superimposed and lines a, b, c, and d, cut into the brightfield negative to mark the limits of chromosomal detail, as indicated.
The marked negative B is then placed in the mask C so that the significant grain patterns fall into the clear path of width W. This width represents the maximum value for the anaphase and telophase figures analyzed. The mask C is an integral part of the scanning apparatus shown in fig. 3. (It corresponds to the item labeled (b) there.)
It should be noted that the actual brightfield negative used in the analysis differs from that illustrated by figure B in color relationship: its grains are light transmitting areas on a dark background.

telophase. For the compilation of the data, these areas were expressed in terms of the weight of paper comprising them and the two values thus obtained were transformed into a single value, their ratio.

Figure 5 shows the graphical distribution of the ratios obtained from the analysis of 30 anaphase and telophase cells. The ratios vary from 1.0:1.0, corresponding to a symmetrical distribution of grains in the autoradiographic emulsion (cf. Plate I, figures 2a and 2b) to 2.5:1.0, corresponding to extreme asymmetry, with more than twice as many grains over one half than over the other (cf. Plate I, figures 1a and 1b).

Before the conclusion of an unequal distribution of newly

synthesized DNA between the products of chromosomal duplication becomes acceptable, it is necessary to take a closer look at the significance of the measured inequalities and find an answer to the question as to whether the newly synthesized DNA could be equally distributed and still lead to the same results. In other words, could the measured inequality be due to experimental error?

The data obtained are subject to three major types of error:

a. The number of grains in the autoradiographic emulsion may not be strictly proportional to the amount of new DNA synthesized.

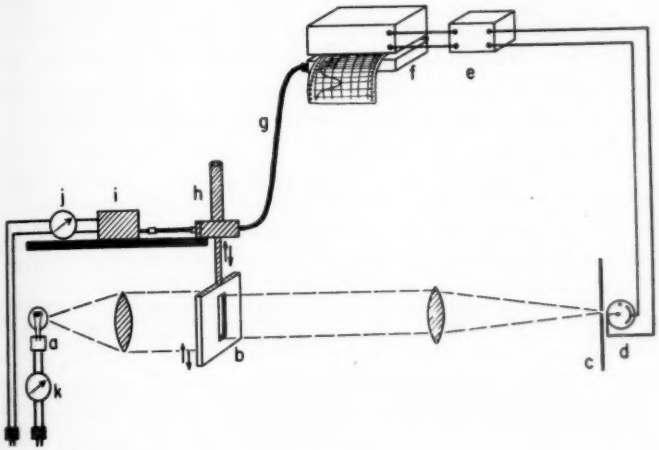

FIG. 3. Diagram of Scanning Apparatus.

a—light source;
b—negative holder and mask;
c—phototube slit, end view;
d—gas-filled phototube;
e—impedance matching device;
f—amplifier and recorder (Hazotrol Corporation, Palo Alto, Calif.; Servo unit HC 100 and chart drive HC 10, operated through flexible shaft (g) by motor (i), thus synchronized with negative holder (b));
g—flexible drive shaft;
h—device for vertical movement of negative holder;
i—variable speed motor;
j and k—variacs.

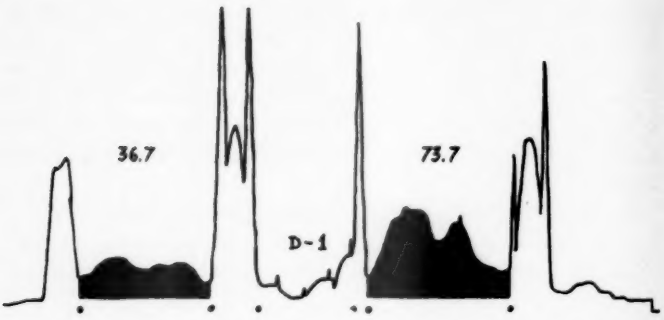

FIG. 4. Sample scanning record of a telophase autoradiograph. The jagged peaks are the positions of the cuts in the negative. The shaded areas are the measure of light transmission due to the two halves of the telophase. Their weights, in mg, are noted. The resulting ratio: $\frac{73.7}{36.7} = 2.0{:}1$.

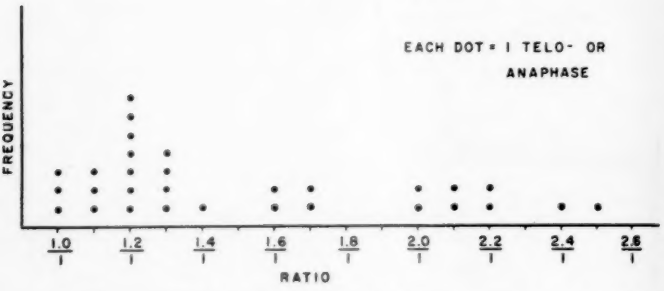

FIG. 5. Frequency distribution of the ratios obtained from the 30 anaphases and telophases measured. (Ratio values rounded off to nearest tenth unit.)

b. There may be an imperfect geometrical relationship between the radiation source—the chromosomes—and the radiation detector—the emulsion.

c. The quantitation procedure is subject to some error.

It is not possible at the present time to evaluate all of these errors perfectly. A careful consideration of their possible and probable effect lead us to the conclusion that the inequality observed is real and biologically significant, that the results obtained are not consistent with the hypothesis of the equal distribution of newly synthesized DNA. (For a detailed discussion of errors see Plaut and Mazia, 1956.)

The most probable conclusion is, therefore, that there occur unequal amounts of new DNA in the products of one nuclear division, that one set of daughter chromosomes may have a different amount of new DNA from its sister set. The possibility of significant variation in total DNA between the two chromosomal groups in a postmetaphase cell appears very remote. Photometric studies both in *Crepis capillaris* (Plaut and Mason, unpublished) and other material (Patau and Swift, 1953) furnish no basis for such an assumption. We can conclude then, that the proportion between newly synthesized DNA and old DNA is not necessarily the same for the products of one nuclear division. This conclusion stands in opposition to the suggestion of Daoust *et al.* (1954) that DNA synthesis involves the reconstitution of all the DNA. With respect to thymidine, at least, we can differentiate between two "types" of DNA, new and old, labeled and unlabeled. Insofar as the behavior of the DNA fraction of the chromosome is typical of the behavior of the chromosomal material as such, we have an answer to the initial question on chromosome reproduction: the parental material is not equally distributed between the daughter chromosomes.

The alternative hypothesis is that the parent chromosome remains intact and a new unit, identical to the parent, is synthesized during reproduction. It is very probable that this statement represents an oversimplification, that what remains intact is not the whole chromosome as such but a subdivision thereof. In other words, it is very likely that the pre-duplication chromosome consists of more than one independent unit of synthesis. This conclusion, in the present context, is not based on the microscopic appearance of the chromosome but on the distribution of the ratios of new DNA in post-metaphase cells obtained in this study. If chromosomal reproduction involves the synthesis of an entirely new unit and the experimental analysis is restricted to a single chromosome, we would expect only one type of new: old distribution in the post-metaphase cell: one half should have all the new DNA, the other only old, unlabeled DNA, if the unit of synthesis is the chromosome itself. If, on the other hand, the chromosome is comprised of two independent units and two new units are synthesized, we may get one of two distributions; all new: all old, or one half new and one half old on each side,

depending on the segregation of the units. If we assume random segregation of the units of synthesis, the two distributions would be expected to occur with equal frequency. These same considerations apply to more complex models, with more chromosomes and more independent units per chromosome. Figure 6 illustrates the increase in expected distribution types as the number of chromosomes and the number of subunits increases.

For the purpose of obtaining some idea of the number of independent subunits in the *Crepis capillaris* chromosomes, we have calculated the expected distribution types and their frequencies for a six chromosome system with varying numbers of subunits per chromosome, and compared the results with those actually observed. Without assuming a very high degree of accuracy for

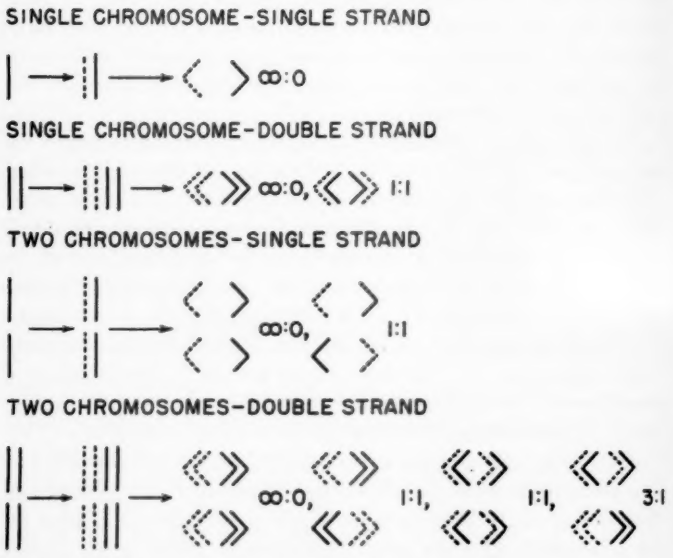

FIG. 6. The consequences of label distribution among chromosomal duplication products with varying numbers of chromosomes and independent subunits. The ratio to the right of each figure represents label distribution. These models are based on the hypothesis that the process of duplication involves the synthesis of new units and not the equal partition of the old. The dotted lines correspond to newly synthesized, the solid lines to old units.

individual ratio values, it appears improbable that the data are consistent with the idea of a single independent unit per anaphase chromosome and very improbable that there are more than three such units per chromosome. If the data are taken literally, they show very good agreement with the expectations from a three unit chromosome. Whether this latter estimate proves to be meaningful or not depends on the amount of correction to which individual ratio values are subject and on the validity of several simplifying assumptions which have been made (see Plaut and Mazia, 1956, for further discussion).

The demonstration that chromosomal duplication does not involve the equal partition of parental DNA between the two daughter chromosomes is obviously not enough to permit the postulation of a mechanism of chromosomal replication. The establishment of the number of independent units of which one chromosome consists, as attempted speculatively above, is one further step in that direction. Another obvious necessity is the study of other chromosomal constituents. On the other hand, we can use the conclusion of unequal distribution of new DNA as an experimental test of one of the current hypotheses on the duplication of the DNA molecule: the DNA duplication scheme suggested by Watson and Crick (1953). According to this theory, the two double helices resulting from the duplication of one double helix would both consist of one half new and one half old material. If every DNA molecule of a chromosome duplicated according to this scheme, we would expect the DNA molecules in the two daughter chromosomes to be equally new and old. Since this is not found to be the case in our experiment, we must conclude that every DNA molecule in the chromosome cannot replicate according to the Watson-Crick mechanism. Whether only a portion of the DNA molecules of the chromosome undergo replication or whether replication does not involve the permanent separation of the double helix cannot be decided at the present time.

Acknowledgments

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fying the scanning apparatus to its present stage, to Dr. Spencer Brown for providing supplies of *Crepis capillaris* seeds, and to Dr. M. Friedkin for generously supplying us with samples of C^{14}-thymidine.

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NUCLEOTIDE METABOLISM AND CANCER CHEMOTHERAPY (Abstract)

HOWARD E. SKIPPER*

It is not uncommon to hear somewhat emotional debates among those engaged in cancer research concerning the relative merits of two approaches to the advancement of the status of cancer chemotherapy: (1) empirical screening of large numbers of compounds against various animal and human neoplasms, versus (2) searches for exploitable biochemical differences between normal and neoplastic cells as an essential prerequisite to the planned synthesis of selection of compounds with greater selective toxicity to disseminated cancer cells in man.

It appears obvious to this author that both approaches are complementary. An attempt will be made to correlate some results obtained by use of both approaches.

Within the time available the following topics will be discussed: (1) the theoretical approach to selective toxicity, (2) effects of temporary anticancer agents on nucleotide metabolism, (3) searches for exploitable biochemical differences between normal and neoplastic cells, and (4) the present stumbling blocks to more effective cancer chemotherapy.

* Kettering-Meyer Laboratory, Southern Research Institute (affiliated with Sloan-Kettering Institute), Birmingham, Alabama.

THE EFFECT OF ANTIPYRIMIDINES ON NUCLEIC ACID METABOLISM

ARNOLD D. WELCH*

The studies to be described have been concerned with three main areas: (a) the metabolism of orotic acid and the design and synthesis of analogs of it for antagonism of the utilization of this important intermediate in the biosynthesis of pyrimidine-containing nucleotides; (b) the metabolism of uracil and the design and synthesis of analogs of it for applications similar to those described in (a); and (c) the metabolism of thymine and its deoxyriboside, thymidine, and the design and synthesis of analogs of them for specific interference with the biosynthesis of deoxyribonucleic acid (DNA).

(a) Among various compounds synthesized by Greenbaum in these laboratories, several have exhibited activity for inhibiting the growth of strains of *Lactobacillus bulgaricus* on orotic acid. Particularly active in this respect have been 6-uracil sulfonamide, 6-uracil methyl sulfone and the corresponding benzyl sulfone; 6-uracil sulfonic acid was without such activity. From studies of the mechanism of inhibition of partially purified enzyme systems by Holmes, evidence has been obtained for a primary competitive intereference by the sulfonamide or the methyl sulfone with the phosphoribosylpyrophosphate (PRPP)-dependent enzymatic conversion of orotic acid to the ribotide, orotidine-5′-phosphate. However, the characteristics of this inhibition change promptly from competitive to non-competitive kinetics; the latter circumstance is in agreement with findings obtained with growing *L. bulgaricus* 09, on which bacterial species these analogs produce an inhibitory effect that is not prevented by an excess of orotic acid (or uracil, uridine or uridine-5′-phosphate (UMP)). The possibility is afforded that the inhibitory analogs are converted to the corresponding ribotides and that these derivatives are responsible for the non-competitive inhibition, however, the validity of this hypothesis has not yet been established. In any case, it can be assumed that the affinity of the antagonist,

* Department of Pharmacology, School of Medicine, Yale University

or a derivative of it, for one or more enzymes concerned with pyrimidine metabolism, is very great, so that displacement is not readily attained. In intact mice both the methyl and benzyl sulfones exert a toxic effect; also, the former compound inhibits the growth in mice of sarcoma-180 and of the lymphomas L-1210, L-4946 and L-5178, while the benzyl sulfone is nearly inactive in this respect (Hakala, Law and Welch). Whether this relative inactivity of the latter compound is related to differences in the physiological disposition of the two compounds or to differences in the mechanisms of the antibacterial and the antitumor activities is not yet known.

(b) Since it is now clear that certain mammalian cells, as well as many strains of bacteria, are capable of deriving nucleic acid-pyrimidines not only from orotic acid, but also from free uracil (Canellakis; Leibman and Heidelberger; Reichard; Paschkis *et al.*) it is of interest that analogs of uracil such as 5-azauracil and 6-azauracil, studied by Canellakis and Handschumacher, respectively, in these laboratories, are able to interfere with uracil utilization, at least by various bacterial species. The latter compound inhibits the growth of strains of *L. bulgaricus* 09, *Streptococcus faecalis*, *L. casei*, *L. leichmannii*, and *Pediococcus cerevisiae* (*Leuconostoc citrovorum*). The action of the analog on these bacterial species is competitively antagonized by uracil. It is remarkable, however, that for a uracil-requiring mutant of *E. coli*, 6-azauracil is without inhibitory activity; it may be of interest, therefore, to determine whether the mechanism by which uracil is utilized by this organism is remarkably different from that of 6-azauracil-susceptible species.

With *S. faecalis* it can be demonstrated (Handschumacher) that maximal effectiveness of the antagonist is attained in the presence of amounts of folic acid or thymine which are in excess of those required for optimal growth in the absence of the antagonist. Strains of *S. faecalis* selected for resistance to 6-azauracil, unlike the parent strain, do not effectively utilize exogenous uracil, although the capacity for the assimilation of uridine and deoxyuridine appears to be unaltered in the resistant variants. This finding is suggestive of possible mechanisms of active transport of uracil and 6-azauracil by these cells and studies of this feature and of the activity of 6-azauridine and the corresponding

deoxyriboside will be carried out. Chemical synthesis of the former is under investigation, while enzymatic formation of the latter has been demonstrated with *S. faecalis* cells incubated with thymidine in the presence of an excess of 6-azauracil. Preliminary studies of the mechanism of inhibition of bacterial growth strongly suggest that incorporation into nucleic acids may not be responsible for the interruption of reproductive activity, and studies of the possible role of 6-azauracil in inhibiting the formation of coenzymes, *e.g.*, uridine diphosphate-containing compounds, are in progress.

Studies of the effect of 6-azauracil on the growth of various neoplasms of the mouse (with Hakala and Law) have demonstrated its inhibitory activity for sarcoma-180, lymphomas L-1210, L-4946 and L-5178, and also for mammary adenocarcinoma 755. Although the effect on survival time is not marked, inhibition of the growth of the solid tumors is worthy of further investigation. These effects are obtained without evidence of significant toxicity for the host, and chronic toxicity studies in monkeys (Schmidt), using doses as high as 90 mg. per kg. per day (in three divided doses), have disclosed no evidence of toxic effects on any organ or tissue, including the hematopoietic tissues and the intestinal epithelium. In puppies, however, doses of 30 mg. or more per kg. per day cause anorexia and evidence of disturbances of intestinal function.

That the intestinal epithelium of rats is capable of utilizing free uracil for nucleic acid synthesis has been shown by Leibman and Heidelberger, and a similar utilization by slices of rat or mouse liver has been demonstrated in our laboratories by Canellakis. The work of the latter indicates strongly that earlier studies in intact animals (Plentl and Schoenheimer; Brown *et al.*) which indicated that isotopically labeled uracil cannot serve as a nucleic acid-precursor, were subject to error because the absolute amounts used were insufficient to saturate the very active degradative system of the liver. Canellakis has found that a TPNH-dependent system of the liver converts uracil to the 5,6-dihydro-derivative; this, following hydrolytic opening of the ring, is converted to β-alanine, ammonia and carbon dioxide (derived from carbon-2 of the uracil). This highly active catabolic system, which is absent from the intestine, is not blocked by the aza-

uracils. In order for uracil to be incorporated into ribonucleic acid (RNA) by the liver, the amounts introduced into the animal must be sufficient to exceed the capacity of the rate-limiting first step in the catabolic sequence found in this organ; however, when this is accomplished, uracil is incorporated efficiently into RNA. It is of considerable interest that carcinogen-induced hepatomas of rats have been reported (Paschkis *et al.*) to incorporate uracil-2-C^{14} into RNA, under conditions in which incorporation into normal liver was not observed. Since Canellakis has found that the active uracil-catabolizing system of normal mouse liver is of minimal activity in spontaneous hepatomas in this species, while RNA-synthesis from uracil-2-C^{14} is unimpeded in the neoplasm, a possible explanation for the earlier findings may be afforded.

(c) A third area of investigation, directed toward specific interference with the biosynthesis of DNA, has involved study of the mechanism of action of 6-azathymine as an inhibitor of growth in various bacterial species (e.g., *L. casei*, *L. leichmannii*, and *S. faecalis*). Reminiscent of the situation which obtains with 6-azauracil and the uracil-dependent mutant of *E. coli*, is the finding that a thymine-requiring mutant of *E. coli* is not inhibited by 6-azathymine. Studies by Prusoff in our laboratories have indicated that *S. faecalis* converts 6-azathymine to the deoxyriboside (azathymidine) and that the latter, an even more active antagonist, may be responsible for the inhibitory action of azathymine. For an organism such as *Thermobacterium acidophilus* (R-26), which utilizes thymidine, but not thymine, azathymidine is a potent antagonist, while azathymine is essentially inert.

When *S. faecalis* cells are exposed from the time of incubation of the medium to radioactive azathymine, in a concentration insufficient to inhibit bacterial reproduction completely, azathymine can be demonstrated in the bacterial DNA; under these conditions the uptake of the analog is equivalent to a replacement of from 12 to 16 per cent of the DNA-thymine. On the other hand, when sufficient labeled azathymine is added to *S. faecalis* during its exponential growth phase to cause a prompt arrest of bacterial growth, gross incorporation into bacterial DNA does not occur. Thus, a more specific type of block may be involved, *e.g.*, interference with the formation or function of a specific type of DNA

or of a hypothetical thymidine-containing coenzyme has been suggested by Prusoff.

Using mammalian cells, *e.g.*, rabbit bone marrow and Ehrlich ascites tumor cells, azathymidine (but not azathymine) appears to interfere with the utilization of thymidine for the biosynthesis of DNA, as evidenced by the incorporation of formate-C^{14} into DNA-thymine.

Although the deoxyriboside of azathymine is exceedingly difficult to synthesize biologically, various studies of its activity are now in progress. These involve not only the possibility of a selective inhibitory action on the reproduction of certain neoplastic cells, but also the proliferation of certain viruses known to be rich in DNA. It is of interest to record that azathymidine though excreted rapidly by the kidneys, at least in the mouse, is apparently essentially insusceptible to nucleosidases or to the influence of other catabolic enzyme systems. Although the problem of chemical synthesis of deoxyribosides remains unsolved at present, there is reason to be optimistic concerning eventual progress along these lines, particularly if the practical utility of such a compound as azathymidine could be demonstrated. Accordingly, investigations of the biological activity of this and related substances will continue.

As an outgrowth of these studies, recent work (Prusoff *et al.*) has shown that the biosynthesis of DNA-thymine from formate-C^{14} by Ehrlich ascites tumor cells is significantly increased by the addition not only of deoxyuridine or uridine, but even more markedly by the addition of deoxycytidine or cytidine; also, orotic acid serves to increase the uptake of formate-C^{14} into the thymine of DNA. This work has encouraged new studies of the intermediates involved in the biosynthesis of the precursors of DNA-thymine.

CALLING ATTENTION TO

Items of Possible Interest to Medicos

September, 1956

Russian Physiology: Its comprehensiveness, largely in the Pavlovian canon, is well shown in *Communications at the XXth International Congress of Physiologists in Bruxelles* (USSR Academy of Sciences, Moscow, 1956, 392pp, Russian, French, German & English). LA Orbeli has new Institute of Evolutionary Physiology in Leningrad, & has issued 1st vol of series on *Materials of Evolutionary Physiology* (USSR Academy of Sciences, Leningrad, 1956, 320pp in Russian). Institutes are large, well equipped, and with keen staffs. Kulteshi section of Pavlov Institute of Physiology out from Leningrad, has superb animal facilities and broad comparative program.

XXth International Congress of Physiology at Brussels: 2800 attending crowded sessions. *Proceedings* in 2 parts: *Reviews* (541 pp. of symposium sessions) & *Abstracts of Communications* (1062 pp). Symposia on neuronic inhibition & excitation, initiation of sensory discharge; hypothermia; water & food intake; acid secretion by stomach; striated & smooth muscle; cardiac muscle; reticular formation of brain; structure & function of brain; psychopharmacology, & hypothalamus and pituitary. Exhibit on *Some Founders of Physiology* (Am. Physiol. Soc., Washington, 1956, 122pp) approached subject comprehensively. Special exhibit on Belgian physiology. 1959 Congress in Buenos Aires.

Psychophysiology & pharmacology: W Bevan & Co show taming has little effect on audiogenic convulsions (*Science* 124/74/July 13/56). GM Everett & Co discuss drug tremor & its antagonism (*ibid* p 79). AE Fisher reports maternal & sexual behavior induced by intracranial chemical stimulation (*ibid* p 228/Aug 3/56). DX Freedman proposes apomorphine test for tranquilizing drugs (*ibid* p 264/Aug 10/56). J Olds & Co use self-stimulation of brain for screening tranquilizers (*ibid* p 265). SK Roberts finds "clock" controlled activity rhythms in fruit flies (*ibid* p 172/July 27). E Shaw & DW Wooley show serotonin action of LSD (*ibid* p 121, July 20), & report on antiserotonins in hypertension (*ibid* p 34/July 6/56).

Clinical: H Begemann edits *Proc. 5th Congress European Society for Hematology* (Springer, Berlin, 56, 921pp, DM 148). J Brozek edits *Body Measurements & Human Nutrition* (Wayne State Univ Press, Detroit, 56, 176pp, $3.5). PF Hahn edits *Therapeutic Use of Artificial Radioisotopes* (J Wiley, NY, 56, 414pp, $10). EW Hurst & R Hall discuss chemotherapy of virus diseases (*Pharmacol. Rev.*, 8/199/56). HL Rosonoff shows hypothermia causes decrease in cerebral blood flow & metabolism, brain vol, & electrical & reflex activity (*Surg* 40/328/56). H Pepper & S Lindsay find increase in blood platelets as indication of ovulation (*Science* 124/180/July 27). MS Sadove & Co give clinical appraisal of trifluor-ethylvinyl ether ("Fluormar") following report (*J Pharmacol* 108/488/53) of JC Krantz & Co (*Anesth* 17/591/56).

Preclinical: J Axelrod proposes mechanism of tolerance to narcotic drugs (*Science* 124/263/Aug 10/56). I Banga & Co differentiate metacollagen from elastin (*J Gerontol* 11/242/56). RW Berliner & J Orloff discuss carbonic anhydrase inhibitors (*Pharmacol Rev* 8/137/56). VE Cosslett discusses limitations of electron microscopy (*Endeavor* 15/153/56). RI Dorfman & RA Shipley review *Androgens: Biochemistry, Physiology & Clinical Significance* (Wiley, NY, 56, 590pp, $13.5). F Eichholtz well handles *Die Toxische Gesamtsituation auf dem Gebiet der menschlichen Ernahrung* (Springer, Berlin, 56, 178pp, DM 22). PJ Flory considers role of crystallization in polymers & proteins (*Science* 124/53/July 6/56). S Graff edits *Essays in Biochemistry*, in honor of Hans T. Clarke (Wiley, NY, 56, 345pp, $6.5). T Hayashi discusses *Chemical Physiology of Excitation in Muscle & Nerve* (Nakayama Shoten, Tokyo, 56, 166pp, $3). CC Lindegren discusses stability of the gene (*Science* 124/26/July 6/56). CC Pfeiffer generalizes on optical isomerism & pharmacological action (*ibid* p 29). D Rudnick edits *Cellular Mechanisms in Differentiation & Growth* (Princeton Univ Press, 56, 375pp, $7.5). LA Woods reviews nalorphine (*Pharmacol Rev* 8/175/56).

General: MG Arnstein reviews Florence Nightingale's influence on nursing (*Bull NY Acad Med* 32/540/56). ER Hilgard offers 2nd ed, well revised, of *Theories of Learning* (Appleton, NY, 56, 563pp, $5.5). JR Murlin & B Kramer well survey long quest for anti-diabetic hormone (*J Hist Med* 11/288/56).

OCTOBER, 1956

Clinical: PO Astrand surveys human physical fitness with reference to sex & age (*Physiol Rev* 36/307/56). J Boberg-Ans describes methods of estimating corneal sensitivity (*Acta Ophthal* 34/149/56). CS Cameron edits symposium on care of elderly cancer patients (*Geriatrics* 11/365–412/56). CJ Clemedson reviews blast injury (*Physiol Rev* 36/336/56). RV Ford & Co find mecamylamine (ganglionic blocking agent but not quarternary ammonium cmpd) has long action, is absorbed orally, & is best in moderate or severe hypertension (*Am J Med Sci* 232/129/56). RF Rushmer reviews structure & function of cardiac ventricles (*Physiol Rev* 36/400/56). H Van Cauwenberge shows adrenocortical atrophy after cortisone is prevented by giving salicylates (*Arch Internat Pharmaco* 106/473–599/56). G Whittembury & Co show no pulmonary blood increase in mitral stenosis (*Acta Physiol Lat-Amer* 6/15/56).

Brain: FM Berger & Co find benactyzine (diethyl-aminoethyl-benzilate, "Suavitil") an antispasmodic with no taming effect, but with slowing action on cortex (*Proc Soc Exp Biol Med* 92/563/56). MK Howitt suggests wide variations in biology of schizophrenia (*Science* 124/429/Sept 7/56). MH Jones shows notion of "2nd pain" is artifact (*ibid* p 442). DL Keller & WW Umbreit show reserpine clears chemically altered behavior in fish (*ibid* p 407/Aug 31). D Krech & Co show increased spatial discrimination with increased cholinesterase activity in cerebral cortex (*J Comp Physiol Psychol* 49/261/56). H McLlwain surveys electrical phenomena & speed of chemical change in brain (*Physiol Rev* 36/355/56). EJ Walaszek & LG Abood use Siamese fighting fish to test tranquilizing drugs (*Science* 124/440/Sept 7/56).

Preclinical: GH Bishop outlines natural history of nerve impulse (*Physiol Rev* 36/375/56). HE Carter edits symposium on nucleic acids (*Fed Proc* 15/811–848/56). V Erspamer & Co show repeated injections of high doses of 5-hydroxytryptamine give tolerance & renal cortical degeneration (*Arch Internat Pharmaco* 106/122/56). I Feber & Co find absorption of glucose in gut requires phosphatase action & normal ATP level (*Acta Physiol Acad Sci Hung* 10/19, 33/56). E Hintzsche offers *Das Aschenbild tierischer Gewebe und Organe* (Springer, Berlin, 56, 141pp, DM 40).

G Keighley & Co find plasma from anemic animals contains polycythemic factor (*Science* 124/439/Sept 7/56). PK Knoefel & KC Huang study biochemorphology of renal tubular transport, showing x-ray visualization of urinary system by iodized benzoates only if cmpds are excreted by tubules (*J Pharmacol* 117/307/56). J Knoll climbs on conditioned reflex band-wagon in showing corical radiation from active focus on stimulation for environment scanning continues until motor reaction is completed (*Acta Physiol Acad Sci Hung* 10/89/56). MH Lietzke confirms TF Young's prediction that weight-lifting ability is proportional to two-thirds power body weight (*Science* 124/486/Sept 14/56). HT Meryman discusses mechanics of freezing cells (*Science* 124/515/Sept 21/56). RW Moncrieff shows smell discrimination depends on ordorant adsorption on olfactory receptors with resulting adaptation (*J Physiol* 133/301/56). JT Mountain & Co find vanadium pentoxide 100 ppm in food lowers liver cholesterol & phospholipid (*Proc Soc Exp Biol Med* 92/582/56). H Theorell discusses nature of oxidation enzymes (*Science* 124/467/Sept 14/56). CC Toh shows alkali extracts of kidney has factor ("nephrosin") releasing serotonin & histamine from platelets (*J Physiol* 133/402/56). T Velasquez & WO Fenn find adaptation to low CO does not change ability of muscle to burn CO to CO_2 (*Acta Physiol Lat-Amer* 6/23/56).

General: M Calvin discusses chemical evolution & origin of life (*Am Sci* 44/248/56). M Espinasse offers biography of microscopist Robert Hooke 1635–1703 (Univ California Press, Berkeley, 56, 204pp, $4). HM Evans gives worthy appreciation of JMD Olmsted 1886–1956 (*Science* 124/395/Aug 31/56). R Foregger reports argument between Jan IngenHousz (1730–1799) & J Priestley (1733–1804) on CO_2 absorption (*Anesth* 17/511/56). WF Libby gives full data on radioactive strontium fall-out & its consequences (*Proc Nat Acad Sci* 42/365/56). ER Long gives *A History of Tuberculosis & the Case of Frederic Chopin* (Univ Kansas Press, 56, 71pp, $2). JM Luck edits symposium on jubilee of American Society of Biological Chemists (*Fed Proc* 15-793/56). GN Plass notes long range effects of CO_2 accumulation on climate (*Amer Sci* 44/302/56).

NOVEMBER, 1956

Clinical: American Psychiatric Asso makes *An Evaluation of the Newer Psychopharmacologic Agents* (Psych. Res Rep #4, Am Psych Asso, Washington 6 DC, 56, 129pp, $2). HB Atlee discusses *Natural Childbirth* (CC Thomas, Springfield, Ill, 56, 79pp, $2). MC Cobey analyses *Postrual Back Pain* (*ibid*, 88pp, $3). JH Davis & WE Abbott consider changing concepts in pathology of thermal burns (*Surg* 40/788/56). FF Foldes describes *The Use of Muscle Relaxants in Anesthesia* (CC Thomas, Springfield, Ill, 56, 244pp, $5.5). N

Leyton writes on *Headaches: The(ir) Reason & The(ir) Relief* (*ibid*, 150pp, $2.5). FE Mohs proposes *Chemosurgery in Cancer, Gangrene & Infections* (Ibid, 314pp, $14.5). JR Rodger advises judicious use of amphetamines for sleepy auto & truck drivers, to help avoid accidents (*GP* 14/90/56). EH Rubin considers *The Lung as a Mirror of Systemic Disease* (CC Thomas, Springfield, Ill, 56, 312pp, $12.5). MS Schulzinger analyzes *The Accident Syndrome: The Genesis of Accidental Injury: A Clinical Approach* (CC Thomas, Springfield, Ill, 56, 260pp, $6.5). LM Shefts gives *The Initial Management of Thoracic & Thoraco-Abdominal Trauma* (*ibid*, 121pp, $4). GN Thompson edits broad discussion on *Alcoholism* (*ibid*, 560pp, $9.5). CV Weller reviews *Causal Factors in Cancer of the Lung* (Another Thomas item, 128pp, $3).

Preclinical: H Berneit & Co propose nomenclature for enzymes of fatty acid metabolism (*Science* 124/614/56). GA Brecher gives evidence of ventricular diastolic suction (*Circulation Res* 4/513/56). He also well analyzses *Venous Return* (Grune & Stratton, NY, 56, 158pp, $6.5). HE Carter edits symposium on nucleic acids (*Fed Proc* 15/811/56). LV Heilbrunn well considers *The Dynamics of Living Protoplasm* (Academic Press, NY, 56, 327pp, $6.5). DL Keller & WW Umbreit cause lasting behavior change by chemical & psychological means (*Science* 124/723/Oct 19/56). JM Kinney & Co propose method of estimating carbon balance (*Surg* 40/16/56). HR Mahler & Co describe enzymatic oxidation of urate (*Science* 124/705/Oct 19/56). SE Mayer & JH Bain localize fluorescent acridine convulsants in cell nuclei & nuclear membranes, & with same cmpds localize blood-brain barrier between outer wall of capillary endothelium & glial plasma membrane (*J Pharmacol* 118/1, 17/56). RW Moncrieff analyses olfactory adaptation & odor likeness (*J Physiol* 133/301/56). WJ Nickerson & G Falcone identify disulfide reductase as cellular division enzyme (*Science* 124/722/Oct 19/56). LF Peltier & Co study fat embolism (*Surg* 40/657, 665/56). A Pirie & R van Heyninger survey *The Biochemistry of the Eye* (CC Thomas, Springfield, Ill, 56, 331pp, $7). SRM Reynolds & VJ DeFeo note menstrual delay after reserpine (*Science* 124/726 Oct 19/56). RW Ryall finds chlorpromazine less anti-histaminic than promazine & notes their quinidine-like action (*Brit J Pharmacol Chemoth* 11/339/56). P Shubik & J Sico conclude there is no tolerance level for carcinogens (*Cancer Res* 16/728/56). WF von Oettingen gives basic data on *Acute Toxicities* (Vol 1 Hndb Toxicol, WV Saunders, Phila, 56, 415pp, $7).

General: HL Arnold Jr reviews centennial of Hawaii Medical Asso (*Hawaiian Med J* 15/313/56). Now available is A Einstein's *Investigation on the Theory of Brownian Movement* (Dover, NY, 56, 122pp, $1.25). SB Hendricks de-describes control of growth & reproduction by light & darkness (*Amer Sci* 44/229/56). Hans von Hentig analyses *Der Desperado: Ein Beitrage zur Psychologie des regressiven Menschen* (Springer, Berlin, 56, 236pp, 20DM). CN Hinshelwood & L Pauling eulogize Amedeo Avogadro 1776–1865 (*Science* 124/708/Oct 19/56). V Johnson pays worthy tribute to AJ Carlson 1875–1956 (*ibid*, p 713). RT Kelleher studies intermittent conditioned reinforcement in chimpanzees with applications to importance of money in human behavior (*Science* 124/479/Oct 12/56). LM Klauber tells about *Rattlesnakes: Their Habit, Life Histories & Influence on Mankind* (Univ

Calif Press, Berkeley, 56, 2 vols, 1530pp, $17.5). J Lederberg reviews genetic transduction (*Amer Sci* 44/264/56). HS Liddell analyses *Emotional Hazards in Animals & Man* (CC Thomas, Springfield, Ill, 56, 97pp, $2.5). JM Luck edits symposium on golden jubilee of Amer Soc Biol Chemists (*Fed Proc* 15/793/56). S Polsky compiles *The Medico-Legal Reader* (Oceana Publ, NY, 56, 256pp, $1). AR Ruskin beautifully brings together *Classics in Arterial Hypertension* (1st vol in WW Nowinski's American Lecture Series on Classics in Science & Medicine, CC Thomas, Springfield, Ill, 56, 400pp, $9.5).

C. D. Leake

RAT THYROID ANTIBODIES: LOCALIZATION AND EFFECT ON BASAL METABOLISM OF THE INJECTED RAT*

LUDWIK ANIGSTEIN, G. W. EKLUND AND DOROTHY M. WHITNEY

In the present study, an attempt has been made to influence the physiological responses of the host by the action of specific antibodies. It was found previously that the injection of anti-organ immune serum into a host is followed by phenomena primarily due to the selective affinity of antibodies for the homologous organ in addition to extensive cross-reactions with heterologous organs as recorded by various authors (Pressman and Sherman, 1951; Bale and Spar, 1954; Anigstein *et al.*, 1954). The resulting antigen-antibody combination can be demonstrated *in vivo* by the uptake of labeled gamma globulin with higher radioactivity concentration in the homologous organ. As the result of the antibody fixation, profound metabolic changes were induced in rats injected with antipancreas immune serum as demonstrated by a significant hyperglycemia (Anigstein *et al.*, 1954). It is possible that the insulin of the rat pancreas acted as antigen and produced an anti-insulin component in the serum of the immunized rabbit.

Recent extensive studies by Witebsky on antigenicity of thyroid extracts have greatly contributed to the establishment of the serological pattern of thyroid antisera (Witebsky, Rose and Shulman, 1955). In continuing our studies on thyroid antibodies labeled with I 131, a compound was produced which exhibits a double thyrotropism, namely that of iodine with its inherent affinity for the thyroid, and that of the organ specific thyroid antibodies. After injecting labeled rat-thyroid antibodies into rats, the localization of radioactivity in the various organs was explored and the effect of the antibodies on the metabolic rate of the rat was measured.

Previous preliminary experiments in this line have shown that rats injected with rat-thyroid antiserum exhibit a relatively low oxygen consumption, thus indicating the possible influence of the specific antiserum on the basal metabolic rate of the rat.

(Anigstein *et al.*, 1956). This phase of the work was continued and further elaborated in the present study.

Material and Methods

(a) *Immune serum and radioactivity.* In the preparation of thyroid antiserum, the technique previously described was followed (Anigstein *et al.*, 1956). Male albino rats were injected intra-abdominally with Nembutal-sodium (45 mg./kg.) and with heparin sodium solution and then perfused with saline. After all tissues appeared bloodless, the thyroid gland was removed and either frozen or homogenized in 5 ml. saline in a glass tissue homogenizer. For each experiment, thyroids of 5 to 6 rats were used and 2 rabbits (2–3 kg.) were injected intra-muscularly or intra-abdominally twice a week over a period of 3 weeks using each time 2.5 ml. of the homogenate per rabbit. On other occasions 0.1 ml. was injected intravenously twice weekly. This applies to fresh or frozen and thawed thyroid tissue. After 3 weeks of immunization procedures and one week of rest, the rabbits were bled, serum collected and kept in frozen state until used.

In the preparation of gamma globulin, precipitation was done with sodium sulfate, as exemplified by the following procedure: To 2.0 ml. serum in a 50 ml. round bottom large-necked centrifuge tube add 10 ml. sodium sulfate solution (18.5 gm./100 ml.) and 25 ml. ether, shaking vigorously. Allow to stand at room tempterature for 10 minutes. Add to the ether layer, detergent (Span-20) in a concentration of about 1 per cent. Shake vigorously, centrifuge at 10,000 r.p.m. for about 15 minutes. Upon the removal of the tubes the gamma fraction at the water-ether junction appears as a hard white wafer. The latter is removed and dissolved in 10 ml. saline. The protein solution was dialysed against 16 liters of saline by rotary dialysis at 5–10° C. for 24 hours to remove as much sulfate as possible. The content of the cellophane tubing was then transferred to a large round bottom flask for lyophilization. Radioiodination of the gamma globulin was carried out by Abbott Laboratories by Gleason's technique. The specifications for the various compounds used were as follows:

Globulin No. 139. Anti-rat-thyroid.	
Gamma globulin purity by paper electrophoresis	80 per cent
Assay in microcuries	251 UC/ml.
Specific activity in microcuries	93 UC/mg.
Concentration of globulin in solution	2.7 mg./ml.
Radioactive globulin solution per rat	0.5 ml.
Microcuries per rat	125 UC

Globulin No. 138. Anti-rat-thyroid.	
Gamma globulin purity by paper electrophoresis	85 per cent
Assay in microcuries	236 UC/ml.
Specific activity in microcuries	86.7 UC/mg.
Concentration of globulin solution	2.72 mg./ml.
Radioactive globulin solution injected per rat	0.5 ml.
Microcuries per rat	118 UC

Globulin No. 145. Anti-rat-thyroid.	
Gamma globulin by chromatogram	100 per cent
Assay in microcuries	175 UC/ml.
Specific activity	104 UC/mg.
Concentration of globulin solution	1.53 mg./ml.
Radioactive globulin solution injected per rat	0.75 ml.
Microcuries per rat	130 UC
Normal rabbit serum globulin.	
Gamma globulin purity (paper electrophoresis)	90 per cent
Assay in microcuries	193 UC/ml.
Specific activity in microcuries	96 UC/mg.
Concentration of globulin solution	2 mg./ml.
Radioactive globulin solution injected per rat	0.5 ml.
Microcuries per rat	96 UC
Normal rabbit gamma globulin.	
Gamma globulin by chromatogram	100 per cent
Assay in microcuries	374 UC/ml.
Specific activity in microcuries	90 UC/mg.
Concentration of globulin solution	3.36 mg./ml.
Radioactive globulin solution injected per rat	0.5 ml.
Microcuries per rat	187 UC

Preparation of blood and tissue samples for radioactivity measurement was done in the same manner as previously described, except that 2N NaOH was used in the place of 1N NaOH for digestion of tissues. Geiger-Mueller TGC2 tube with Tracerlab 64 "Scaler" was used for measurement of the grade of radiation emitted from radioiodine in blood and tissues. Counts per minute per gram of wet tissue were corrected for background and for dosage based on 250 gm. rat.

Serological tests of the immune serum included complement-fixation and precipitin tests based on the techniques described by Witebsky (1955). The complement-fixation procedure employed the use of 0.1 ml. each of the components involved, fresh tissue antigen, buffered saline, and 2 units each of complement and hemolysin. The incubation time was 3 hours, 2 hours at 4° C. followed by 1 hour at 37° C., after which sensitized cells were added, and the test read when the controls showed hemolysis.

The standard ring test for precipitin reactions was used by layering the antigen (tissue homogenate), in various dilutions, on the undiluted serum. The titers of the antisera varied from 1:100 to 1:1000.

(b) *Oxygen consumption.* In our previous studies on thyroid function, individual rats were placed in a glass jar dessicator to which a respirometer with a flow of oxygen was attached. The consumption of oxygen was then registered by kymograph recording drum, and the direct measurements of the volume consumed were registered (Anigstein, Hardin, and Whitney, 1956). Higher accuracy was provided in the present work by the apparatus originally designed by Holtkamp, Ochs, Pfeiffer, and Heming (1955) for the determination of oxygen consumption in rats, and modified by Panos, Finerty,

and Wall (1955). This is essentially a closed circuit respirometer permitting the direct measurement of the volume of oxygen consumed by 8 individual rats.[1] A constant temperature of 28° C. is maintained by a thermostatically controlled heating unit in a water bath aided by a small circulating pump. Each animal is kept in a quart Mason jar supplied with oxygen from a bellow (Fig. XI).

Experimental and Results. Radioactivity Measurements

The present experiments were designed with the purpose of exposing the rat thyroid to the action of the specific antiserum followed by the subsequent intracardial injection of radioactive gamma globulin fractionated from anti-rat-thyroid immune serum. By intracardial injection, the radioactive antibodies are transmitted instantaneously via the blood stream to the various rat organs, reaching their ultimate destination in the respective tissues. Comparative control experiments were conducted by pretreating rats with normal rabbit serum and subsequently injecting them with labeled gamma globulin fraction of normal serum (Series 4 and 6).

The experiments were conducted on 6 series of rats as follows: *Series 1:* Twelve rats were divided in 2 groups of 6 each. Rats of one group were injected intra-abdominally with 0.5 ml. anti-rat-thyroid serum; one group was not injected. Four hours later, both groups were injected intracardially with 0.5 ml. radioiodinated anti-rat-thyroid gamma globulin. Thereafter, one rat from each group was perfused daily and tissues prepared for counting over a period 6 days. *Series 2 and 3:* Twenty-four rats were divided into 2 groups of 12 each. Rats of one group were injected intra-abdominally with 0.5 ml. anti-rat-thyroid serum; the other group were not injected. Four hours later, all 24 rats were given intracardially. 0.5 ml. radio-iodinated anti-rat-thyroid gamma globulin. For 6 consecutive days, 4 rats (2 from each group) were perfused and tissues prepared for counting. *Series 4:* Twelve rats were divided into 2 groups of 6 each. One group was injected intra-abdominally with 0.5 ml. of pooled normal rabbit serum; the other group of 6 rats were not pretreated. Four hours later, each rat of the series was injected intracardially with 0.5 ml. I 131 normal gamma globulin. One rat from each series was perfused and tissues prepared for counting over a 6 day period. *Series 5:* Twelve rats were divided into 2 groups of 6 each. One group was injected intra-abdominally, each rat receiving 0.5 ml.

anti-rat-thyroid immune serum. Four hours later, both groups (12 rats) were injected intracardially with 0.5 ml. I 131 anti-rat-thyroid gamma globulin. Rats were perfused at a rate of 2 daily (1 from each group), and tissues prepared for counting for 6 consecutive days. *Series 6:* Eighteen rats were divided into 2 groups of 9 each. One group was injected intra-abdominally with 0.5 ml. normal rabbit serum. Four hours later, all rats were injected intracardially with 0.5 ml. I 131 normal rabbit globulin. Two rats (1 from each group) were perfused daily and the tissues prepared for counting for a period of 9 days.

The two-phase operation was followed as in previous studies with the time of 4 hours elapsing between the two injections. This was done in view of previously reported results with the anti-pancreas serum, when an almost immediate response of the injected rat with development of hyperglycemia, was observed (Anigstein *et al.*, 1954).

In the series of rats given I 131 anti-rat-thyroid gamma globulin, the ratios of radioactivity in the thyroid of the pretreated rats remained on a low level for the first 2–4 days and then rose considerably for the remaining period of observation. The non-pretreated group showed a continuous rise for the first 3–5 days followed by lower levels of radioactivity (Figs. I and VI). These findings suggest a possible functional suppression of the rat thyroid followed by a higher uptake of radioactive iodine for the remaining period.

In the control series composed of rats re-injected with I 131 normal gamma globulin, both the pretreated and the non-pretreated rats showed a parallel increase in radioactivity for the first 4 days followed by lower ratios, the decrease being more marked in the non-pretreated group (Figs. I and VI). It seems therefore that no suppression of the thyroid occurred in the normal globulin series. This is supported by a greater uptake in radioactivity as expressed in tissue/blood ratios in the series involving anti-rat-thyroid gamma globulin as compared with normal gamma globulin series (Figs. I, V, VI). This observation indicates higher retention of antithyroid gamma globulin in the thyroid tissue as compared with gamma globulin of normal rabbit sera.

Earlier studies on tracing anti-organ antibodies *in vivo* have revealed extensive cross reactions with heterologous organs. The

TABLE I

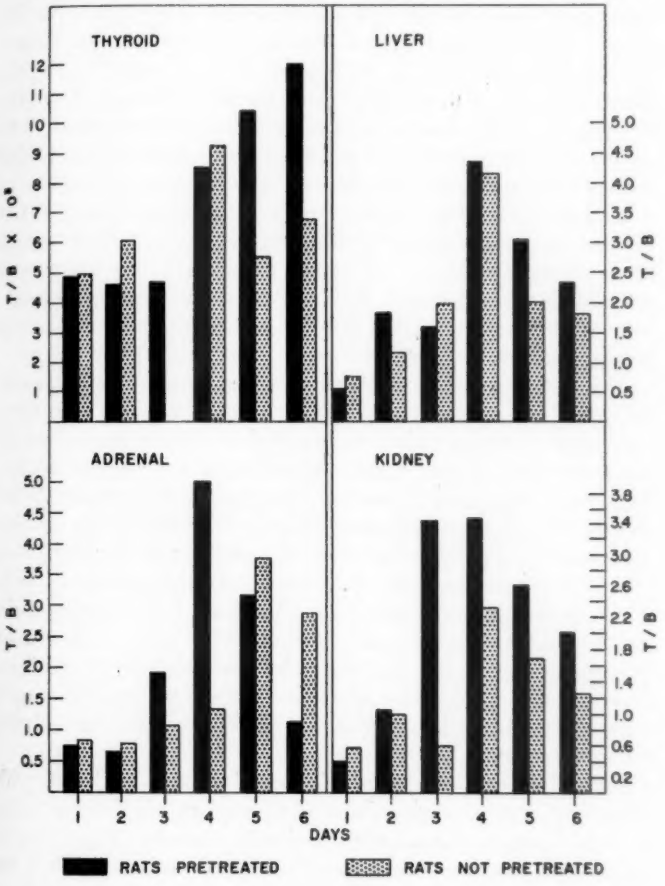
Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-rat-thyroid gamma globulin injected into rats.

Series 1. Fig. I.

Group A—Rats pretreated with 0.5 ml. anti-rat-thyroid serum.
Group B—Rats not pretreated.

	T/B RATIOS Group A	DAY	T/B RATIOS Group B
THYROID:	4818	1	4889
	4451	2	6038
	4626	3	2891
	8409	4	9090
	10165	5	5408
	11907	6	6619
ADRENAL:	.730	1	.882
	.651	2	.791
	1.963	3	1.050
	5.106	4	1.379
	3.218	5	3.734
	1.116	6	2.975
LIVER:	.512	1	.764
	1.763	2	1.150
	1.584	3	1.973
	4.257	4	4.051
	2.988	5	1.878
	2.270	6	1.760
KIDNEY:	.393	1	.595
	1.801	2	1.031
	3.562	3	.954
	2.599	4	2.393
	2.699	5	1.764
	2.027	6	1.242

FIG. I SERIES I
RADIOACTIVITY EXPRESSED IN TISSUE/BLOOD RATIOS (T/B) FOR LABELED ANTI-THYROID GAMMA GLOBULIN. IN TISSUE OF RATS



effect and uptake of anti-thyroid antibodies by heterologous organs was simultaneously studied in adrenals, kidney and liver. In analyzing the graphs showing radioactivity values in the adrenals we found a stepwise increase in T/B ratios for both groups of rats until the fourth or sixth day with no demonstrable suppression of radioactivity. A parallel increase in T/B values was also recorded in the non-pretreated rats with the peak being delayed for one or two days as compared with pretreated rats (Figs. I–IV). In series 4 and 6 (Figs. IV and VI) of rats treated with normal rabbit sera an increase of radioactivity was noted during the entire 6 days of observation, indicating a longer retention of radioactivity in the adrenal tissues than in the homologous thyroid.

In the kidney of rats treated with the anti-rat-thyroid serum there is a varied degree of radioactivity accumulation ranging from a steady increase (Series 5, Fig. V) to an abrupt peak reached on the third day, followed by a steady drop in radioactivity. The same variations of the T/B ratios and the resulting pattern apply to both groups of rats.

The radioactivity of the liver in series 2 and 3 (Figs. II and III) shows relative constant levels for both groups of rats, however relatively high values were noted in series 1 and 5, reaching their peaks on the fourth and sixth days (Figs. I and V). This was particularly evident in the group of rats previously injected with anti-thyroid serum. Generally speaking, the concentration of radioactivity in thyroid, adrenals and kidney was higher in the group of rats pretreated with the anti-serum than in the non-pretreated rats. This was true of all the organs in series 1. In series 5, the peak of radioactivity values was delayed as compared with the non-pretreated animals (Fig. V). These findings may suggest some inhibition in the capacity of the organ to combine with antibody in a host previously injected with the immune serum.

The difference between the T/B ratios of rats injected with anti-thyroid gamma globulin and normal rats is demonstrated by logarithmic radioactivity elimination curves (Fig. VII). It was shown that the elimination from the thyroids of rats of the anti-thyroid series progressed at a high rate for the first 3 days, followed by a leveling off period (Series 1). Conversely, the elimination rates for thyroids of normal gamma globulin control

series (Series 4) showed from the beginning a constant low level followed by a gradual decline. This would suggest a relatively low retention capacity for the homologous gamma globulin by the thyroid tissue in rats of the antithyroid series.

Rats assays for basal metabolism (BMR)

The present experiments were based on 3 series of male rats, namely: one group of 16 rats (Series 1); one group of 16 rats (Series 2): one group of 14 rats (Series 3). The rats were 2 months old and the average weight per rat for all three series was 169 at the outset of the experiment. An average gain in weight at the conclusion of the study was 35.5 gm. per rat in all series. Each series of 8 rats was divided in 2 groups: Group A was injected intra-abdominally with anti-rat-thyroid immune serum, whereas rats of group B were injected with normal rabbit serum. The antiserum and the normal serum were diluted with 0.9 per cent saline 1:1, and 0.5 ml. injected. All injections were given 3 times a week for a period of 2 weeks.

Preceeding all injections, oxygen consumption determinations were made for each animal. Four rats from each group to be injected with normal serum or antithyroid serum were placed in the individual Mason jar oxygen consumption chambers. The chambers were then connected to the oxygen tubes from their respective bellows, and placed in the constant temperature water bath (Fig. XI). The rats were allowed to breathe pure oxygen for a period of about 30 minutes before any measurements of oxygen consumption were made. By the end of this period of acclimatization, the rats were resting quietly. Three consecutive readings for oxygen consumption were then made at 15 minute intervals. The average of these readings were then taken, and the c.c. 0_2/gm./hr. for each rat was determined. All rats were then injected, one group with normal serum and the other with antithyroid serum.

The basal oxygen consumption ratios of the test rats injected either with antithyroid serum or with normal rabbit serum were evaluated and compared with values for normal untreated rats of the same age. The latter data supplied by Richard L. Wall (personal communication, 1957), indicated a characteristic curve for the normal untreated rats (Fig. X-C), showing a gradual decrease in oxygen consumption during the observation period.

TABLE II

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-rat-thyroid gamma globulin injected into rats.

Series 2. Fig. II.

Group A—Rats pretreated with 0.5 ml. anti-rat-thyroid serum.
Group B—Rats not pretreated.

	T/B RATIOS Group A	DAY	T/B RATIOS Group B
THYROID:	29	1	33
	14	2	212
	94	3	95
	32	4	328
	162	5	123
	234	6	228
ADRENAL:	.359	1	.552
	.533	2	.669
	.776	3	.633
	.687	4	1.053
	.929	5	.748
	.779	6	1.054
LIVER:	.444	1	.513
	.397	2	.532
	.402	3	.258
	.418	4	.420
	.375	5	.465
	.442	6	.553
KIDNEY:	.485	1	.455
	.602	2	.712
	.673	3	.631
	.375	4	.866
	.686	5	.671
	.902	6	.811

FIG. II SERIES 2
RADIOACTIVITY EXPRESSED IN TISSUE/BLOOD RATIOS (T/B) FOR LABELED ANTI-THYROID GAMMA GLOBULIN IN TISSUE OF RATS

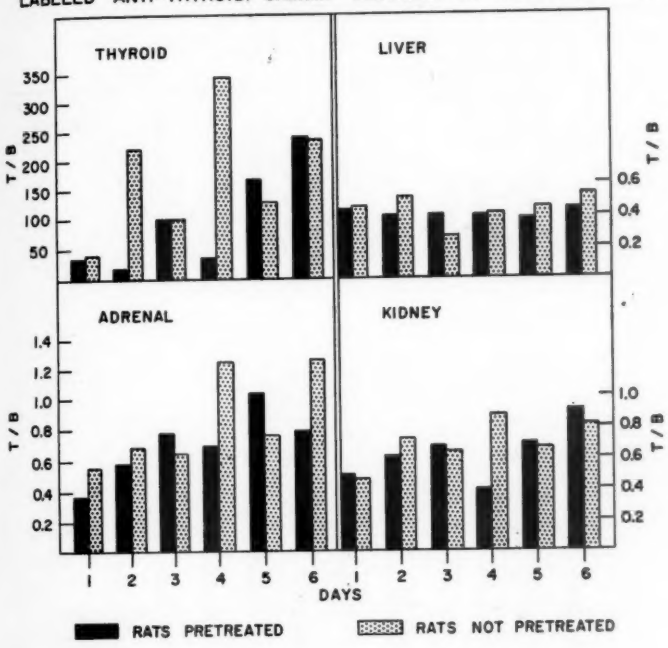


TABLE III

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-rat-thyroid gamma globulin injected into rats.

Series 3. Fig. III.

Group A—Rats pretreated with 0.5 ml. anti-rat-thyroid serum.
Group B—Rats not pretreated.

	T/B RATIOS Group A	DAY	T/B RATIOS Group B
THYROID:	49	1	49
	54	2	86
	295	3	173
	155	4	56
	264	5	220
	135	6	177
ADRENAL:	.314	1	.351
	.653	2	.359
	.563	3	1.219
	1.187	4	1.066
	1.533	5	1.848
	.600	6	1.412
LIVER:	.366	1	.373
	.371	2	.642
	.412	3	.692
	.665	4	.601
	.715	5	.793
	.360	6	1.127
KIDNEY:	.667	1	.413
	.590	2	.335
	.279	3	.680
	.550	4	.767
	.913	5	1.189
	.753	6	1.310

FIG. III SERIES 3

RADIOACTIVITY EXPRESSED IN TISSUE/BLOOD RATIOS (T/B) FOR LABELED ANTI-THYROID GAMMA GLOBULIN IN TISSUE OF RATS

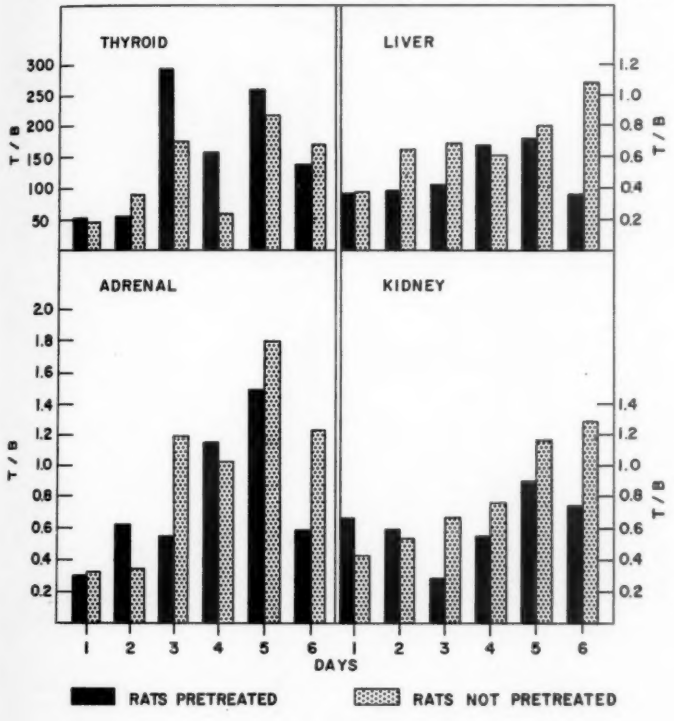


TABLE IV

Radioactivity expressed in tissue/blood ratios (T/B) for labeled normal rabbit gamma globulin injected into rats.

Series 4. Fig. IV.

Group A—Rats pretreated with 0.5 ml. normal rabbit serum.
Group B—Rats not pretreated.

	T/B RATIOS Group A	DAY	T/B RATIOS Group B
THYROID:	1389	1	816
	2778	2	1453
	4020	3	4527
	6807	4	6456
	6613	5	4355
	6158	6	2952
ADRENAL:	.679	1	.745
	1.217	2	.871
	1.020	3	3.343
	1.660	4	1.938
	2.938	5	1.508
	4.830	6	2.606
LIVER:	.786	1	.641
	.587	2	.808
	1.084	3	1.230
	.945	4	2.027
	2.700	5	1.962
	2.085	6	1.695
KIDNEY:	.733	1	.745
	.860	2	.750
	1.414	3	2.093
	1.975	4	2.114
	3.717	5	1.802
	2.522	6	2.742

FIG. IV SERIES 4
RADIOACTIVITY EXPRESSED IN TISSUE/BLOOD RATIOS(T/B) FOR LABELED NORMAL GAMMA GLOBULIN IN TISSUE OF RATS

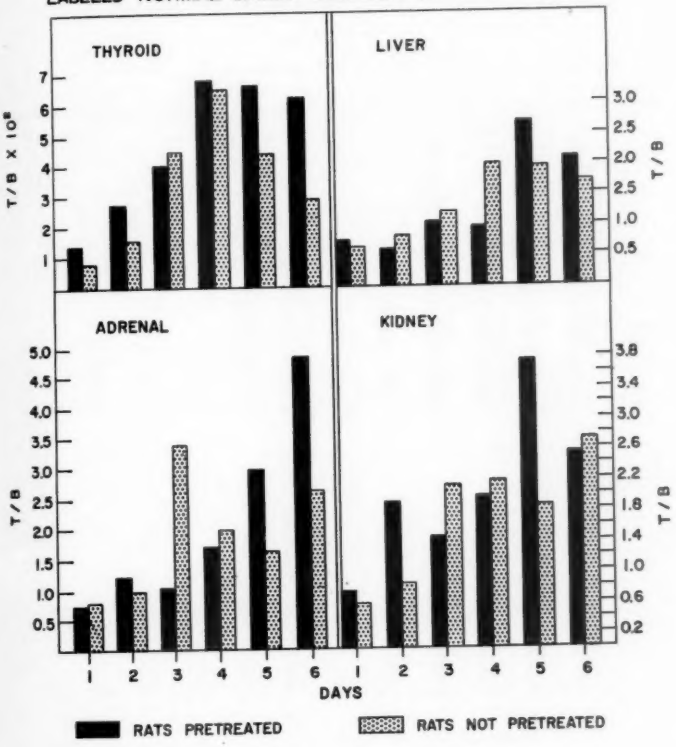


TABLE V

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-rat-thyroid gamma globulin injected into rats.

Series 5. Fig. V.

Group A—Rats pretreated with anti-thyroid serum.
Group B—Rats not pretreated.

	T/B RATIOS Group A	DAY	T/B RATIOS Group B
THYROID:	18.2	1	25.9
	211.8	2	....
	222.4	3	62.4
	192.4	4	102.3
	1,027.5	5	581.7
	466.1	6	335.8
ADRENAL:	.268	1	.313
	.536	2	....
	1.704	3	.609
	1.387	4	.753
	2.511	5	2.816
	3.948	6	2.178
LIVER:	.268	1	.189
	.331	2	....
	.838	3	.567
	.415	4	.497
	.918	5	2.055
	1.110	6	.699
KIDNEY:	.379	1	.303
	.605	2	....
	1.175	3	.624
	1.479	4	.699
	1.901	5	2.580
	3.362	6	1.288

FIG. V SERIES 5

Radioactivity expressed in tissue/blood ratios (T/B) for labeled anti-thyroid gamma globulin in tissue of rats.

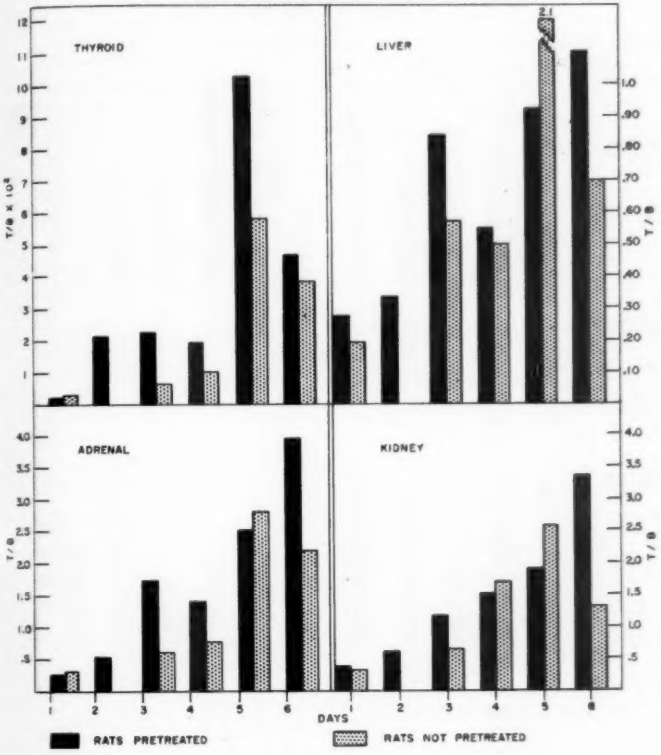


TABLE VI

Radioactivity expressed in tissue/blood ratios (T/B) for labeled normal rabbit gamma globulin injected into rats.

Series 6. Fig. VI.

Group A—Rats pretreated with normal rabbit serum.
Group B—Rats not pretreated.

	T/B RATIOS Group A	DAY	T/B RATIOS Group B
THYROID:	80	1	125
	816	2	273
	707	3	383
	1406	4	1075
	1176	5	811
	541	6	811
	541	6	84
ADRENAL:	.63	1	.17
	.64	2	.32
	.41	3	.34
	.41	4	.28
	.50	5	.45
	.44	6	.56
LIVER:	.121	1	.041
	.069	2	.070
	.042	3	.043
	.077	4	.083
	.078	5	.058
	.053	6	.052
KIDNEY:	.221	1	.102
	.188	2	.220
	.079	3	.143
	.149	4	.181
	.214	5	.386
	.173	6	.131

FIG. VI SERIES 6

Radioactivity expressed in tissue / blood ratios (T/B) for labeled NORMAL rabbit gamma globulin in rats

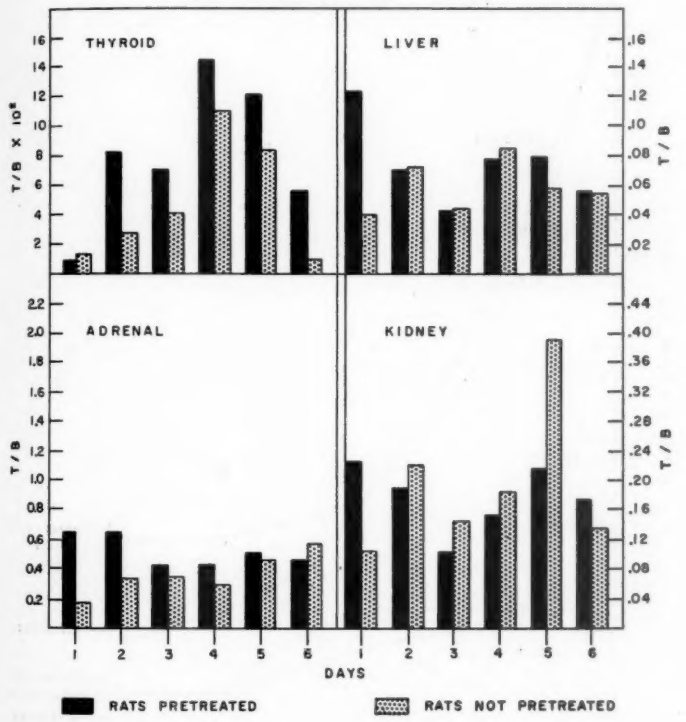


FIG. VII

ELIMINATION CURVES FOR THYROID OF RATS INJECTED WITH LABELED ANTI-RAT-THYROID GAMMA GLOBULIN (SERIES I) AND OF RATS INJECTED WITH LABELED NORMAL GAMMA GLOBULIN (SERIES IV)

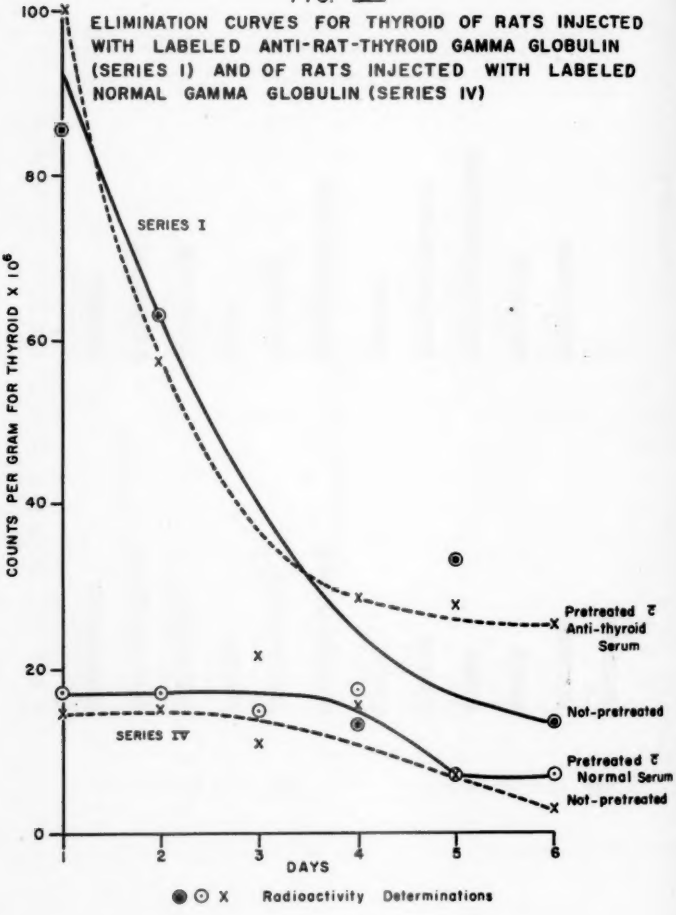


FIG VIII

AVERAGE OXYGEN CONSUMPTION IN RATS TREATED WITH ANTI-THYROID RABBIT IMMUNE SERUM ——A AND WITH NORMAL RABBIT SERUM- - - -B

SERIES 1

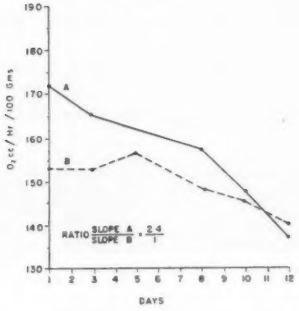


FIG IX

AVERAGE OXYGEN CONSUMPTION IN RATS TREATED WITH ANTI-THYROID RABBIT IMMUNE SERUM ——A AND WITH NORMAL RABBIT SERUM - - - -B

SERIES 2

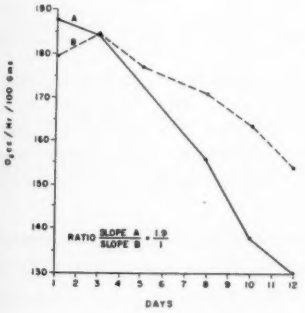
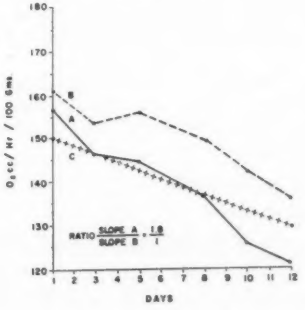


FIG X

AVERAGE OXYGEN CONSUMPTION IN RATS TREATED WITH ANTI-THYROID RABBIT IMMUNE SERUM —— A WITH NORMAL RABBIT SERUM- - - - - - B AND IN UNTREATED RATS + + + + C

SERIES 3



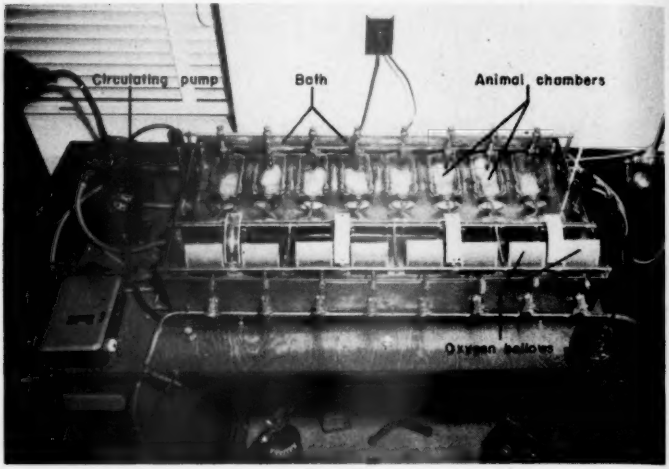


FIG. XI

It can be seen that whereas the curves for series B and C show similar trends in the gradual decrease in O_2 uptake, slope A, representing the antithyroid series shows a sharp decrease in oxygen consumption beginning with the eighth day. This would indicate that the natural downward trend of the oxygen uptake of the growing rat is accelerated by the action of thyroid antibodies. A similar decrease in oxygen consumption can be seen in series 1 and 2 (Figs. VIII, IX, X). The relative values of the curves can be expressed for the slopes A/B of the 3 series of rats in ratios $\frac{1.8}{1}; \frac{1.9}{1}; \frac{2.4}{1}$ in which the denominator is taken as unity to represent the fall in O_2 uptake for the control series B per day. Therefore, it seems that a relative hypothyroid or hypometabolic state has developed in rats under the influence of specific antibodies.

An attempt to interpret the mechanism of this phenomenon leads us to the question as to the essential nature of the thyroid antibodies, namely, whether the action results from a tendency to combine with the tissues of the rat thyroid (anti-organ) or whether they act as antithyroid compounds and can be compared with thyroxine analogues (antihormones). Another interpretation may be that the antibodies might act on the thyroxin

liberated by the thyroid, and, in effect, decrease the amount of active circulating thyroxin, thus decreasing the rate of oxygen consumption. This would increase the TSH (thyroid stimulating hormone) liberation by the pituitary, and thus increase thyroid activity, accounting for greater I 131 uptake.

Uptake of Carrier-free I 131 in Rats

Since the uptake and concentration of labeled antithyroid globulin by thyroid tissue are influenced by the thyrotropism of iodine as well as by the homologous globulin, it was of interest to evaluate the two functions in the following experiment.

Twenty-two male rats were divided in two groups. One group was injected intra-abdominally three times a week for two weeks with 0.5 ml. anti-rat-thyroid serum diluted 1:1 with saline. The other group was injected similarly with normal serum. Twenty-four hours after the last injection, each rat of both groups was injected with 7 microcuries of carrier-free I 131 in 0.7 ml. saline. Following the injection of radioiodine, the animals were sacrificed, the thyroids removed, weighed and digested with 2 N NaOH. The samples were allowed to dry overnight and the radioactivity measured. The average counts per gm./minute for each thyroid of rats treated with normal rabbit serum was 218,329, while the rats treated with anti-thyroid serum showed an average of 361,182 counts/gm./min. in the thyroid tissue. This indicates 1.64 times more activity as compared with the average data for the thyroid of rats treated with normal rabbit serum, indicating a hypo-metabolic state existing prior to the administration of iodine. The greater uptake of I 131 can be interpreted as counteraction to correct the deficiency by promotion of thyroid activity (Chaikoff and Taurog, 1949; Astwood, 1949).

Summary and Discussion

In the current study, the action of anti-rat-thyroid immune serum and of its specific gamma globulin upon the thyroid of the injected rat was further investigated. The demonstration of the thyroid antibodies in rat tissues was made possible by labeling the gamma globulin fraction with I 131. Thus a compound was produced which exhibited a two-fold thyrothropism, namely, that of iodine with its inherent affinity for the thyroid

Group A Treated with anti-rat thyroid immune serum.		Group B Treated with normal rabbit serum.	
Rat 1	258,615	Rat 12	234,667
2	220,634	13	259,666
3	342,917	14	224,429
4	338,333	15	203,706
5	568,750	16	245,186
6	332,091	17	315,722
7	261,000	18	140,000
8	393,563	19	167,000
9	463,000	20	185,000
10	564,154	21	262,143
11	229,941	22	164,095
Average	361,182		218,329

where it synthesizes thyroglobulin, and the homologous antibodies which are attracted and fixed by the specific antigens of the thyroid.

In the preparation of the antisera, thyroid tissue homogenates from 5 to 6 rats were injected into rabbits, twice weekly, intramuscularly, intra-abdominally, or intravenously for a period of three weeks. The immune sera showed titers of 1:100 to 1:1000 by complement fixation and precipitin ring tests with the thyroid antigen. The serum globulin was precipitated by sodium sulfate dialysed and lyophilized. Radioiodination of the lyophilized gamma globulin was carried out by Abbott Laboratories with specific activity of about 100 microcuries per mg. Radioactivity values for the various tissues of the injected rats were expressed in tissue/ blood ratios (T/B) of counts/gm./min.

The influence of thyroid antibodies upon the uptake of labeled gamma globulin was investigated in rats pretreated with a single injection of anti-rat-thyroid immune serum and reinjected with I 131 gamma globulin. The experiments were conducted on a total of 88 rats divided into six series. Each series was composed of two groups of rats, one group injected intra-abdominally either with antithyroid immune serum or with normal rabbit serum. Four hours later, all rats were injected intracardially with either labeled anti-rat-thyroid gamma globulin or labeled normal gamma globulin, and the radioactivity measured in the thyroid and other rat tissues. In rats pretreated with the specific antiserum, low radioactivity (T/B) ratios were noted in the thyroid which suggested a relative suppression of the thyroid function. In the control series of rats injected with labeled normal gamma

globulin both pretreated and non-pretreated groups showed parallel increase in radioactivity of the thyroid indicating no inhibition. Furthermore, it was shown by elimination curves that the radioactivity elimination from rats treated with antithyroid serum progressed at a high rate, contrary to the low level elimination for rats injected with normal rabbit serum.

The radioactivity elimination curves for the thyroid in rats of series 1, injected with labeled anti-rat-thyroid gamma globulin showed, after an initial high radioactivity level, a sharp downswing for the first three days after injection. This applies to the pretreated as well as to the non-pretreated groups until the third day (Fig. VII). At this time a gradual straightening of the curves, especially of rats pretreated with thyroid antiserum was noted. The relatively high concentration of radioactivity in the thyroid of series 1 is probably the result of the combined selective affinity of I 131 coupled with the thyrotropism of the specific antibodies. This excess of radioactivity is rapidly eliminated by the thyroids of series 1, contrary to the low and stationary radioactivity level of the control rats injected with labeled normal gamma globulins (Figs. VII, series 4).

The effect of the anti-rat-thyroid serum on the oxygen consumption and basal metabolic rate was studied on a total of 46 rats. Oxygen consumption was determined in individual rats placed in a closed circuit respirometer of the Holtkamp type apparatus. Each series of rats was divided in two groups, one injected with antithyroid serum and the other with normal rabbit serum. Injections were given three times weekly over two weeks period, preceded by oxygen uptake measurements. The average values were recorded in 0_2/gm./rat/hr. The results indicate that the animals of the antithyroid groups showed a sharper drop in oxygen consumption as compared with that of rats treated with normal serum.

The influence of the thyroid antibodies upon the uptake of radioiodine (carrier-free I 131) was investigated on two groups of eleven rats each: one was injected three times a week for two weeks with anti-rat-thyroid serum, while the other group was injected similarly with normal rabbit serum. Both groups were subsequently injected with seven microcuries of carrier-free I 131 per rat. Radioactivity evaluation showed 1.64 times more radioactivity (counts/gm./min.) in the thyroid tissue of rats

injected with the antithyroid serum as compared with normal serum controls. These results seem to indicate that apparently a hypometabolic state existed prior to the administration of iodine. The greater uptake of I 131 can be interpreted as counteraction to correct the deficiency by promotion of thyroid activity.

Conclusions

1. Gamma globulin of the rat-thyroid antiserum labeled with I 131 and injected intracardially into a rat, localizes primarily in the thyroid gland with extensive cross-reactions in the adrenals, liver and kidney. The radioactivity in the rat thyroid expresses the combined thyrotropism of iodine and of the specific antibodies, carriers of I 131.

2. Pretreating of rats with thyroid antiserum and inoculating them subsequently with labeled antithyroid gamma globulin resulted in a relative suppression of thyroid activity.

3. Pretreating of rats with thyroid antiserum and subsequently inoculating them with carrier-free I 131 resulted in a greater iodine uptake by the thyroid as compared with rats pretreated with normal serum.

4. Rats injected with thyroid antiserum showed lower oxygen consumption rates as compared with intact control rats and those injected with normal serum. This would indicate the possibility of influencing the BMR by the action of thyroid antibodies.

Acknowledgments

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A TECHNIQUE OF SERUM LIPOPROTEIN ESTIMATION AND THE LIPOPROTEIN LEVELS IN RHEUMATIC FEVER*

EDWARD W. BERMES, JR.,† JOSEPH R. CHRISTIAN,‡ AND HUGH J. MCDONALD§

That rheumatic fever is probably of streptococcal origin has been generally agreed upon for some time. It has been shown that group A streptococci are important factors in the pathogenesis of this disease (7). Of the two hemolysins produced by a group A beta hemolytic streptococci, streptolysin S is under suspicion as being responsible for rheumatic fever (8). On the basis of earlier reports that the phospholipid of blood from patients who had exhibited a susceptibility to rheumatic fever was low (8), and that the inhibition of streptolysin S by serum is accomplished by a lipoprotein complex in which phospholipid has a role of primary importance (9), an investigation into the lipoprotein levels of serum from patients with rheumatic fever was undertaken, utilizing the technique of ionography. The alpha-L ratio, which may be defined as the ratio of total lipoprotein to alpha lipoprotein, has been proposed as a relatively sensitive index of disturbances in lipid metabolism (5). The purpose of this study was to investigate a possible alteration in this ratio in cases of rheumatic fever.

Methods and Material

Twelve patients with rheumatic fever, ranging in age from 6 to 16 years were utilized in the study group. Five patients, considered to be "hospital normals," *i.e.*, without clinical evidence of febrile, metabolic or endocrine disease, which might alter the lipoprotein fractions, were utilized as a normal control group.

Blood was obtained from fasting patients and centrifuged in a clinical centrifuge soon after clotting. The serum was then stored at 4° C. until analysis. Ionographic separations of the lipoprotein fractions were then carried out by means of the Precision Ionograph,[1] which utilizes the horizontal open-strip technique (4, 5). The filter paper used to stabilize the buffer solutions was Whatman No. 1, one-half inch in width and 40 centimeters in length. The temperature was maintained constant at 25° C. The buffer employed was veronal at pH 8.6 and ionic strength 0.05. The system was allowed

to equilibrate, with the current on, for one and one-half hours after wetting the horizontal paper strips with the buffer solution and drawing them up taut, and then five micro liters of the serum was applied as a thin streak midway between the ends of the paper. The time of each run was three and one-half hours with a potential gradient of six to eight volts per centimeter. After the separations had been completed, the ionograms were dried in an oven at 100° C. and stored for later staining. All ionograms, except those which were prestained (a procedure which will be described below) were stained by immersion in a saturated solution of acetylated Sudan Black B in 60 per cent ethanol for twenty minutes. The acetylated Sudan Black B was prepared according to the directions given by Lillie and Burtner (2). In preparing the staining solution the alcoholic solution of the dye was heated to boiling then cooled and filtered, first through Whatman No. 1 filter paper, and then through Whatman No. 42 filter paper. Swahn has pointed out that this heating before filtration aids in the removal of small particles of the dye held in suspension in the solution (6).

After the strips were removed from the bath, each one was washed for two minutes in four wash solutions of 50 per cent ethanol. They were then dried and scanned in an automatic scanning device, which has been described in detail elsewhere (5), with the monochromator set at 600 millimicrons, a wave length at or near the maximum absorption of all the blue components of Sudan Black B (1). The areas under the peaks of the alpha and beta lipoprotein curves, which were extrapolated to the base line as previously described (4), were then determined by means of a planimeter (compensating polar planimeter, Keuffel and Esser Company, No. 803).

The pre-staining of lipoproteins was carried out according to a method described earlier by McDonald and Bermes (3). One-tenth of a milliliter of a 500 milligram per cent solution of acetylated Sudan Black B in 95 per cent ethanol was added slowly with shaking to a test tube containing one milliliter of human serum. After thirty minutes the excess alcohol was removed under reduced pressure while allowing a stream of air from a thinly-drawn capillary to blow over the surface of the serum to eliminate frothing. The jet of air also proved to be an efficient means of stirring. After the alcohol had been removed, the serum was centrifuged to remove the excess dye and an aliquot (5 microliters) of the pre-stained serum applied to each filter paper strip. Upon completion of the run the lipoprotein zones appeared blue against a white background. The ionograms were scanned immedittely after drying in the oven since the patterns were observed to fade with time. The areas under the curves were determined with a planimeter. The alpha-L ratios for all the patterns were calculated by dividing the total area under the lipoprotein curves by the area under the alpha lipoprotein curve.

Results and Discussion

In Tables 1 and 2 the alpha-L ratios and their average deviation are given for the sera from twelve children with rheumatic fever and for five samples of serum from normal children. Each

TABLE 1

Alpha L Ratio for Sera From Rheumatic Fever Patients

Case	Age	Sex	Color	Diagnosis	Alpha-L Ratio
1	7	F	W	Acute R. F. without carditis	2.7 ± 0.1
2	10	M	W	Acute R. F. with mitral valvulitis	3.4 ± 0.1
3	16	M	W	Acute R. F. with mitral valvulitis	3.0 ± 0.1
4a.	14	F	N	Acute R. F. with mitral valvulitis	2.7 ± 0.1
4b.	14	F	N	Acute R. F. with mitral valvulitis	2.5 ± 0.2
5	14	F	W	Acute R. F. with mitral valvulitis	2.6 ± 0.1
6	14	F	N	Acute R. F. with mitral and aortic valvulitis	3.5 ± 0.1
7	7	F	W	Acute R. F. with mitral valvulitis	2.8 ± 0.1
8	16	F	W	Chronic R. F. with mitral valvulitis	5.1 ± 0.1
9	12	M	N	Acute R. F. with mitral valvulitis	2.4 ± 0.1
10	13	M	W	Acute R. F. with mitral and aortic valvulitis	2.2 ± 0.2
11	8	M	W	Acute R. F. with mitral valvulitis	2.7 ± 0.2
12	6	F	W	Acute R. F. with mitral valvulitis	5.1 ± 0.2

b—Indicates an aliquot of Serum "a" pre-stained.

TABLE 2

Alpha L Ratio for Normal Sera

Case	Age	Sex	Color	Diagnosis	Alpha-L Ratio
1a.	10	M	N	Normal	2.9 ± 0.1
1b.					2.7 ± 0.1
2a.	8	F	N	Smoke intoxication	3.7 ± 0.1
2b.					3.7 ± 0.1
3a.	9	F	W	Skin abrasions Contusion of nose	4.6 ∓ 0.2
3b.					4.4 ± 0.1
4a.	9	F	N	Fractured humerus	2.9 ± 0.1
4b.					3.0 ± 0.2
5a.	8	M	N	Acute tonsillitis (5-2-55) (Blood studied 5-10-55 after treatment)	2.7 ± 0.1
5b.					2.5 ± 0.2

b—Indicates an aliquot of Serum "a" pre-stained.

ratio is the average value obtained by scanning duplicate inograms. Some of the serum samples were stained by both of the methods described above. In these cases the alpha-L ratio obtained by both methods are given. From these tables it can be seen that there is a considerable variability for the normal sera as well as for the pathological sera. It would appear from this data that there is no obvious correlation between the alpha-L ratio and rheumatic fever.

Summary

On the basis of earlier reports that the phospholipid level of blood from patients who had a susceptibility to rheumatic fever was low, and that the inhibition of streptolysin S by serum is accomplished by a lipoprotein complex in which phospholipid has a role of primary importance, an investigation into the lipoprotein levels of serum from patients with rheumatic fever was undertaken, using the technique of ionography. The fractionation was carried out on a group of serum samples from patients with rheumatic fever and also on a control group of serum samples from normal patients. The experimental conditions were: Whatman No. 1 filter paper, 0.5 inch in width, 40 cm. in length; veronal buffer at pH 8.6, and ionic strength 0.05; potential gradient 6–8 volts per cm.; temperature, 25° C. The inograms were stained for lipoproteins with the specific fat stain, Sudan Black B. The relative amounts of alpha and beta lipoproteins were determined and the alpha-L ratios, *i.e.*, the ratio of total lipoprotein to alpha lipoprotein, determined. From the data obtained it appears that the ratio of total lipoprotein to alpha lipoprotein, which serves as a relatively sensitive index of disturbances in lipid metabolism, is not appreciably altered by rheumatic fever.

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† Standard Oil Foundation Research Fellow, Department of Biochemistry. Supported by the Standard Oil Company (Indiana).

‡ Associate Professor of Pediatrics.

§ Professor of Biochemistry.

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SOME FACTORS AFFECTING THE GROWTH-PROMOTING PROPERTIES OF SERUM IN TISSUE CULTURE*

RELDA CAILLEAU† AND PAUL L. KIRK

Recent techniques for growing cells in tissue culture in direct contact with the nutrient medium without a plasma clot or other substrate have focused attention on the various components present. Many people have modified the growth medium in attempts to achieve one of known chemical composition: Fischer *et al.* (1948), White (1949), Healey, Fisher, and Parker (1954), (1955), and Evans *et al.* (1956a), (1956b). Others have tried to simplify it by using readily accessible stable components which produce uniform results: Morgan, Campbell and Morton (1955), and Waymouth (1956). But the basic growth factors of most media currently used for the continuous cultivation of many cell types is still some form of serum (or ascitic or pleural fluid) and/or embryo extract.

Most people who use a medium containing serum have noted contradictory results, at one time or another, both in their own research and in that reported in the literature. Certain experiments demonstrate that the heating of serum or serum fractions to 56–60° C. will tend to reduce the growth promoting qualities: Carrel and Ebeling (1922), Bazeley, Rotundo, and Buscheck (1954). Other reports indicate that the growth activity of a serum or serum derivative is unaffected by such treatment: Carrel and Ebeling (1922a), Simms, Parshley, and Pitt (1947), Jacquez and Barry (1951).

The present experiments were made in an attempt to explain and understand some of these contradictory results. Many sera, from different species, were tested on several cell strains in an effort to relate the growth promoting or inhibitory quality of a serum, with any specific properties of sera in general.

Materials and Methods

The cells used in these experiments were as follows:

1. Mouse strain L cells[3] (Sanford, Earle, and Likely, 1948) maintained

on a stock medium consisting of 40 per cent horse serum, 40 per cent medium 199 and 20 per cent Tyrode's solution.

2. Mouse liver epithelium cells strain No. A 1469-91-1.[4] These cells have been maintained since April 1954 on medium similar to that above, namely 20–40 per cent horse or ox serum, 40 per cent medium 199 and Tyrode's solution.

3. HeLa cells[5] maintained on medium containing 40 per cent cord, ox or horse serum, 40 per cent medium 199 and Tyrode's solution.

4. Embryonic human muscle and spinal cord cells isolated in this laboratory (Zitcer, Cailleau, and Kirk, 1954), and maintained for several months in continuous subculture in 40 per cent cord serum, 8 per cent EE_{50} and Tyrode's solution.

Stock cultures were kept in T-60 flasks and transferred at frequent intervals depending upon the rapidity of growth. For test purposes, cells were obtained from the stock flasks by trypsin treatment or scraping and seeded into Carrel flasks at a concentration of approximately 200,000 cells contained in 0.1 ml. of suspending fluid (either Tyrode's solution or the test medium). The test medium was added to give a total volume of 1.0 ml. per Carrell flask.

Whenever necessary, cell counts were made as previously described (Cailleau and Kirk, 1954) so that subcultures contained approximately 200,000 cells per Carrel flask. When counts were not made, the growth was estimated visually and dilutions ranging from 1:5 to 1:20 were made to give the approximate concentration for the subculture.

A serum was considered positive only if 4 or more subcultures could be carried out with obvious multiplication of the cells as determined by visual inspection or cell count at the end of the growth period. Any experiment in which no growth occurred was repeated three times with the same serum and with different sources of that type of serum if possible.

The sera used were as follows:

Human cord serum pooled from 4 to 8 sources for each lot of medium tested.

Human serum (adult) pooled from 8 to 15 people.

Monkey serum, from adult rhesus monkeys, usually pooled from 2 or 3 animals.

Horse serum[6] usually pooled from 2 to 4 horses maintained solely for the production of normal serum.

Ox serum[6] pooled from several animals or from the slaughter house from a single animal.

Pregnant mare serum.[6]

Hog serum, 2 different lots, pooled from the slaughter house.

Sheep serum, 2 lots of lamb serum pooled from several animals from the slaughter house; 2 lots of ram, lamb and ewe serum[7] each from 2 or more animals fasted.

Rabbit serum, one lot of pooled serum from adult females, one lot, pooled all males, another lot pooled mixed adults.

Rat serum, three groups of sera were tested; one lot pooled from fasted weanling rats, another from fasted adult rats of both sexes and a third from fasted young male rats (about 9 weeks old). Sera from 3–6 rats were pooled for each group.

Chick serum, from adult cocks, adult hens fasted 48 hours, young cocks fasted overnight, and commercial serum[8] were tested separately. Sera from two or more individuals were pooled for each lot.

Sterility of all sera and total media was assured by filtration through Selas filters and addition of sterile stock solutions of penicillin and streptomycin to produce a final level of 50 units and 50 mg. respectively.

Heated serum was prepared by heating aliquots of the serum in a water bath at a temperature of 56° C. for 30 minutes.

Results

A majority of the experiments reported were carried out with Earle's strain L cells, and to a more limited extent with HeLa and mouse liver epithelial cells. The human embryonic cells gave negative results with all experimental media tested.

The comparative growth of the three main cell strains in the presence of unheated serum is shown in table 1. Both strain L

TABLE 1

Effects of unheated serum on the growth of three types of cells in tissue culture

Serum	strain L cells	mouse liver epithelium cells	HeLa cells
Cord	—	—	+
Human	—	—	+
Monkey	—	—	+
Pregnant Mare	—	—	—
Horse	+	+	+
Ox	+	+	+
Hog	+	?	+
Rabbit	—	—	—
Chicken	—	—	—
Cockeral	—	—	—
Lamb	+	+	+
Ram	+	+	+
Ewe	+	+	+
Rat	+	—	—

cells and liver epithelial cells could not be subcultured regularly when grown in most samples of cord, human, monkey, rabbit or chick serum used. Liver epithelial cells were also unable to grow in rat serum which supported adequate growth of L cells.

On the other hand, HeLa cells grew well in most samples of cord, human and monkey serum used, although certain lots of human sera proved toxic for all types of cells tested. HeLa

cells inoculated in rabbit, chick or rat serum media were not transplantable for more than one subculture in any of our experiments.

Human embryonic muscle and spinal cord cells, while capable of multiplying and undergoing serial subculture on their stock medium (40 per cent cord serum, 8 per cent EE_{50} and Tyrode's solution), could not be maintained for more than one or two subcultures on any other medium tested. Human serum could frequently replace cord serum in the presence of added embryo extract, but toxic batches of human serum were often encountered so that it could not be used as a reliable medium for serial or long term cultivation of these cells.

TABLE 2

Effects of heating sera for 30 minutes at 56° C. on the growth of strain L cells

Serum	Number of subcultures	
	Unheated	Heated
Horse	Unlimited	1–Unlimited*
Human	0–1	2 or more
Cord	0–1	2 or more
Cock	1–2	7 or more
Rabbit	0–3	4 or more

*Cells tend to slip off the glass.

Table 2 illustrates the effect of heating certain sera for 30 minutes at 56° C., upon the serial cultivation of strain L cells. Two diametrically opposite effects were noted, viz. Both inhibition and stimulation of growth promoting properties.

Unheated horse serum which normally provides continuous adequate growth of the L cells (Fig. 1) was partially inactivated

PLATE I

Photomicrographs by Gladys Perez-Mendez of strain L cells grown in Carrel flasks x 150.

FIG. 1. Cells grown in 40 per cent horse serum, 40 per cent mixture 199 and 20 per cent Tyrode's solution. Typical normal looking spindles appear in this medium.

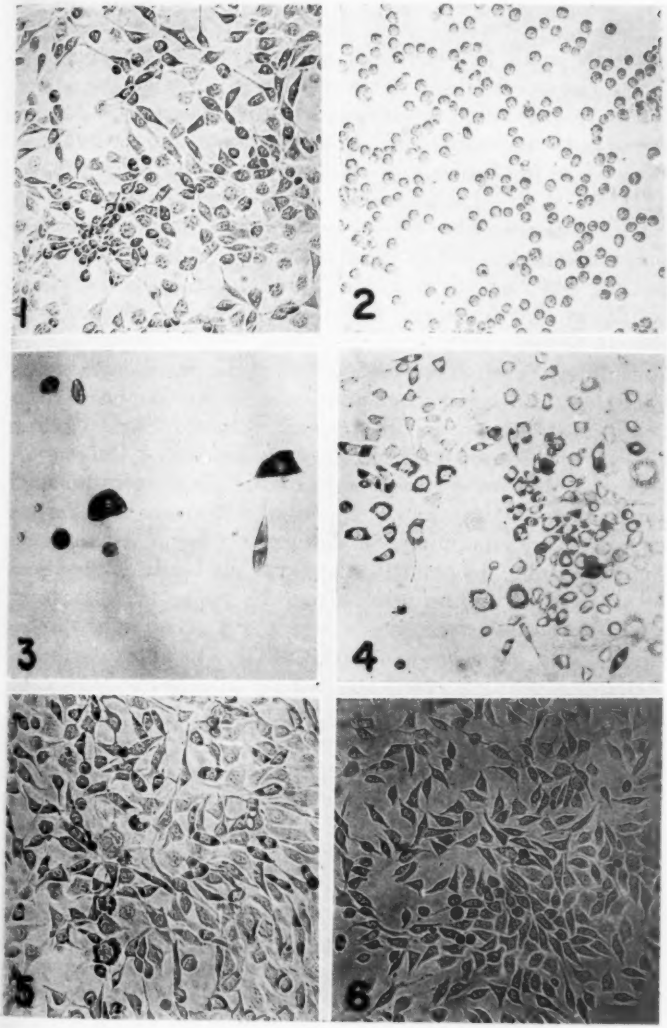
FIG. 2. Growth on same horse serum medium with serum inactivated by heating 30 min. at 56° C. Cells are small rounded, and tend to slough off the glass.

FIG. 3. First culture on media containing cock serum. Cells are heavily granular, rounded or abnormal and can not be subcultured.

FIG. 4. Third subculture on heated cock serum medium. Cells are still granular and abnormal but multiply and assume a more normal appearance.

FIG. 5. Seventh subculture on heated cock serum. Cells nearly normal spindle shaped, some bi-polar granules still present.

FIG. 6. Growth on medium containing 30 per cent normal lamb serum. Typical clear spindle shaped cells.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by heating. A curious effect was observed with two different batches of heated horse serum. The growth of the L cells was usually poor, the cells small rounded and somewhat granular, and they would no longer adhere to the glass as they normally did in the unheated serum. In one series, although the cells no longer spread out on the glass, growth was nearly equal to that found in the presence of the unheated aliquot, and the cells were maintained for 4 subcultures with no change in the growth rate (Fig. 2). With other samples of horse serum, heating was sufficient to destroy some growth factor so that the serum would no longer permit more than 1 to 2 subcultures of the L cells.

Cock and rabbit sera yielded results contrasting with horse serum in that unheated serum was incapable of permitting growth of the L cells while the heated serum allowed repeated subculture. Seven transfers were made in heated cock serum, each subculture being a 1:10 dilution of the previous one. Typical cell counts made on the 5th subculture showed 1.5×10^6 cells at the end of the growth period. The appearance of L cells on cock serum is illustrated in figure 3. In the presence of unheated serum the cells were sparse, abnormal, extremely granular and apparently degenerating. Subcultures could not be made. In figures 4 and 5, the gradual adaption of the L cells to the heated serum is seen. At the third subculture on heated cock serum (Fig. 4) the cells are variable in size and shape, somewhat abnormal and quite granular. By the 7th subculture on heated serum, the cells are practically normal in appearance although many granular forms are still present (Fig. 5).

Sheep serum proved to be a uniformly good growth medium for strain L cells, HeLa and liver epithelial cells. No appreciable difference could be found in the activities of lamb, ram or ewe sera, when they were clear and unhemolysed. One lot of lamb serum, highly hemolysed, produced a granular precipitate and was toxic. Typical growth of L cells in unheated lamb serum is shown in figure 6. Heating all three sera produced a slight deleterious effect and seemed to destroy some of the normal growth promoting properties. Growth of all cell types was poorer in the heated sera, the cells usually being slightly granular, and somewhat rounded or smaller.

The appearance of heavily granular or deeply pigmented cells did not necessarily signify that a serum was toxic or would

not support growth. Figures 7, 8. 9 and 10 illustrate the appearance of the L cells when grown in the presence of rat serum for nine subcultures. In all transplants, the final number of cells varied between 1 and 2 million at the end of the growth period (Table 3), while the cells themselves showed many bizarre, giant

TABLE 3

Multiplication of strain L cells through several subcultures on rat serum and hog serum media

Culture	Number of cells (or dilution used)	
	Rat serum	Hog serum
1	2×10^6	2×10^6
2	1×10^6	1×10^6
3	1×10^6	5×10^5
4	1.6×10^6	8×10^5
5	1.5×10^6	1.8×10^6
6	1×10^6	(not done)
7	(1:10 dil)	2×10^6
8	1.8×10^6	—
9	(1:20 dil)	—

forms, and remained heavily granular throughout all subcultures.

Similar abnormal cells were observed in the presence of rabbit serum. Unheated serum caused the appearance of large, extremely granular degenerating cells (Fig. 11) which did not survive the third subculture. The heated serum, which maintained the L cells for 4 subcultures before being discontinued due to lack of serum, led to somewhat similar abnormalities (Fig. 12).

In the presence of several samples of unheated cord, human, monkey, chick (hen) and rabbit (does) sera, the strain L cells appeared crenated within a few hours. Heated aliquots of these toxic sera permitted some growth for one or two subcultures only. Similar results were found with the liver epithelial cells.

Two lots of hog serum gave results similar to that obtained with rat serum. Continuous subcultures of L cells were maintained for 8 generations, but growth was very slow and many abnormal, large and granular cells appeared. However, cell counts as shown in table 3, indicated continuous multiplication.

In general, markedly hemolysed serum which tended to precipitate in the culture flask was less favorable for growth than

equivalent unhemolysed serum. The most highly toxic sera which caused crenation of the cells in a matter of hours, were those from adult females (chick, rabbit, human) whether the subjects had fasted or not prior to being bled.

Studies on the fatty acid and lipid phosphorus content of certain sera, and the L cells grown in the same serum, indicate that some correlation may exist between the fat content of a serum and its toxic properties (Cailleau, Hoard & Zitcer, 1957).

Discussion

Many of the studies found in the literature on the activity or toxicity of a serum seem to be contradictory. Some of these discrepancies can be resolved when it is known that several factors concerning both cells and sera can influence each result, and must be considered.

Some of the earliest thorough studies on the inhibitory or growth promoting factors in serum were carried out by Carrel and Ebeling (1922a, 1922b, 1923a, 1923b) and Baker and Carrel (1925, 1927). Their experiments utilized fresh chick tissue or 8–10 year old cultured fibroblasts grown in clots and media containing chick embryo juice. Growth was measured during the experimental period but no subcultures were made with the test media. Their studies showed that serum contains both inhibitory and acceleratory factors which vary with the age of the animal. The inhibitory factor increased with age, the acceleratory decreased. The inhibitory factors were found in the lipid fraction and increased when serum was heated 1 hour at 65° C. Heterologous serum was a better culture medium when heated. Serum

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PLATE II

FIG. 7. Second subculture on media containing rat serum. Cells are deeply granular, with many round and abnormal forms.

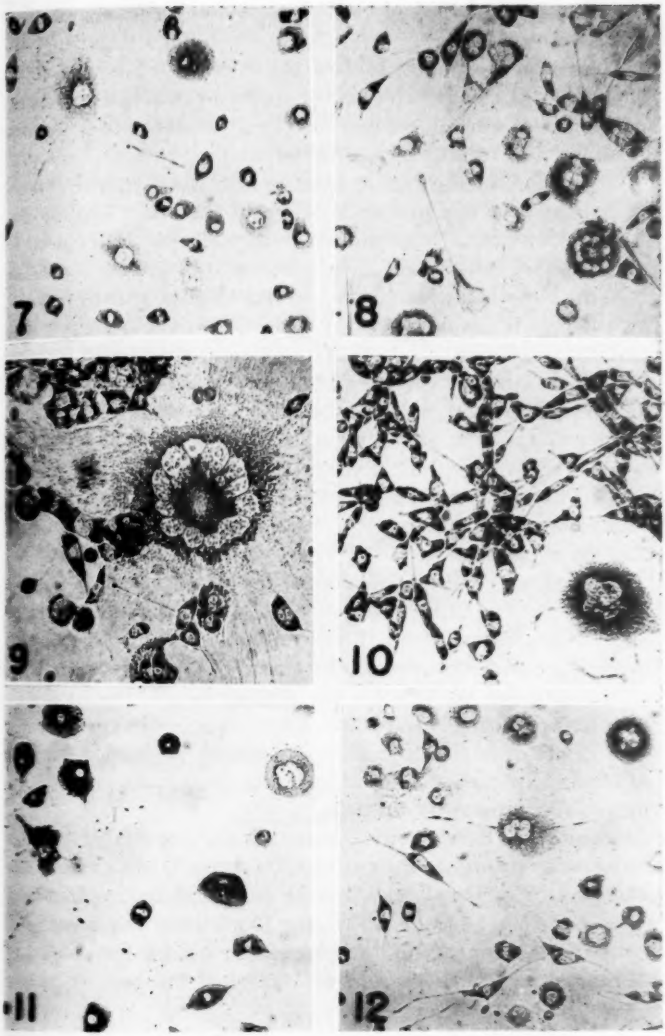
FIG. 8. Fourth subculture on rat serum. Many multinucleate granular cells present.

FIG. 9. Fifth subculture on rat serum. Frequent giant multinucleate cells, and most cells show dark granular pigmentation.

FIG. 10. Eighth subculture on rat serum. Similar deeply granular appearance, now bi-polar as cells become more nearly spindle shaped.

FIG. 11. Third subculture on normal rabbit serum. Cells are extremely granular, abnormal and degenerating. They could not be subcultured.

FIG. 12. Fourth subculture on heated rabbit serum. Cells are mostly granular and abnormal, with many multinucleate forms present, but growth was good and the cells transferable.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globulins seemed to contain the growth factor(s). The accelerator or inhibitory properties of a given serum varied with its concentration.

While our experiments confirm the findings of Carrel *et al.*, in general, the absence of clot and of embryo extract in our media and the use of subcultures and direct cell counts provide greater sensitivity and validity of interpretation.

One or more factors, either toxic or inhibitory, induce clumping crenation or extreme granulation of the cells. Heating the serum to 56° C. for 30 minutes may partially or totally destroy this inhibitory effect and increase the serum's growth potential.

On the other hand, a serum which shows good growth promoting qualities is not improved by heating. On the contrary, it is partially inactivated so that cells grown in the heated fraction are usually smaller, rounded or granular and abnormal when compared with cells grown in the unheated aliquot.

The amount of fat as well as the form in which it is present in the serum may have some influence upon the activity of the serum. The highly toxic sera obtained from adult female rabbits and chickens even though the latter were fasted 48 hours support this hypothesis.

However our results do not necessarily support those of Simms, Parshley and Pitt (1947) who report the presence of lipfanogens and antilipfanogens in sera. These authors indicate that the antilipfanogens, which counteract the effects of the fat forming fraction of sera, are not affected by heating 30 minutes at 60° C. This antilipfanogen activity was found to occur in the albumin fraction of serum. Simms and his co-workers studied fat formation in explants of adult chick aorta fibroblasts in a plasma clot; no subcultures were made.

Jacquez and Barry (1951) studying the non-dialysable factors in cord serum used a strain of rat fibroblasts in a clot, for one generation only. They found that the growth promoting factor(s) was stable when heated to 60° C. for 30 minutes, and was associated with the euglobulin fraction of the serum. Extraction of lipids from dried cord serum did not alter the activity of the serum.

More recently Bazeley, Rotundo and Buscheck (1954) while studying the effects of various plasma fractions on primary cultures of monkey kidney cells noted that certain active fractions

of plasma (fraction V) were relatively stable to temperatures of 60° C. for 4 hours or more. However whole horse or human sera lost over 60 per cent of their growth activity when heated to 60° C. for 2 hours.

The variability of serum as a growth medium was recently emphasized by Chang (1954) in long term continuous subcultures of epithelial-like cells from human tissue. He noted that inactivated sera from different individuals possessed variable growth promoting properties depending upon the cells studied.

Each result reported here is undoubtedly valid for the particular experiment described, but may not necessarily apply to another set of experimental conditions using different cells, media or sera. Carrel and Ebeling's (1923b) inhibitory effects found with adult serum (cat dog and chick) have been confirmed with certain human and chick sera, but we found no measurable difference in the growth of our cells on lamb, ram or ewe serum.

While Baker and Carrel (1925, 1927) and Simms, Parshley and Pitt (1947) found lipids in adult chick serum to be toxic, Jacques and Barry (1951) studying cord serum noted that dried cord serum extracted with benzene, chloroform and ether retained the same activity, when reconstituted, as the untreated aliquot. These authors agreed with Simms *et al.*, in noting that the albumin fraction of serum could counteract the toxic effect of added lipids. We have found cord serum to be uniformly excellent as a growth medium for HeLa cells, while being inadequate for L cells. It has been found essential in media used for maintaining embryonic cells, as adult human serum was found to vary too greatly from one lot to another.

Summary

The ability of serum to act as a growth promoting component in media used for the cultivation of cells *in vitro* is dependent upon several factors.

Each cell type, depending upon its origin, length or method of culture, responds better to sera from certain species than to sera from that of others.

Unheated serum from one species, adequate for one type of cell may be toxic for another. Heating such serum for 30 minutes at 56° C. partially nullifies this toxic effect.

Untreated sera, showing good growth promoting qualities for a given cell strain are adversely affected by being so heated.

In general, sera from young, fasting, male animals seem to contain the best growth promoting properties.

Toxic properties of serum seem to be related to one or more of the following factors: hemolysis, age, fat and possibly sex (female).

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† Present address, Cancer Research Institute, University of California Medical Center, San Francisco 22, California.

[3] Generously provided by Dr. Wilton R. Earle in 1953.

[4] Obtained from Dr. Earle's laboratory through the courtesy of Dr. M. McClain of the Virus laboratory, University of California. This strain is a clone strain of C3H mouse liver cells designated as strain 721 in mixed culture.

[5] Obtained from Cutter Laboratories through the courtesy of Dr. Karol Hok.

[6] Obtained from Cutter Laboratories.

[7] Obtained through the courtesy of Dr. McKircher of the University of California at Davis.

[8] Difco.

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CHEMICAL COMPOSITION OF SOME SERA AND CELLS USED IN TISSUE CULTURES

FATTY ACID AND LIPID PHOSPHORUS CONTENT*

RELDA CAILLEAU,† DOROTHY HOARD, AND ELSA M. ZITCER

The effects of various sera upon the growth of Earle's strain L, HeLa, and liver epithelial cells in tissue culture have been described in detail (Cailleau and Kirk, 1957). In the present study we have attempted to identify some of the factors which might be responsible for the growth-promoting quality of a given serum. Nitrogen, fatty acid and lipid phosphorus determinations were made utilizing micro-methods developed in these laboratories.

Materials and Methods

Tissues or cells studied: Fresh 2½ months old human fetal tissues and serial transfers from these embryonic tissues; Ehrlich's mouse ascites tumor cells; serial cultures of Earle's strain L, HeLa, and liver epithelial cells.

Sera assayed: Cord, human, horse, ox, sheep, monkey, hog, rabbit, rat and fowl.

In general from 2 to 5 aliquots of each tissue, culture, or serum were analyzed at various intervals.

Assay methods: Nitrogen—Grunbaum, Schaffer and Kirk (1952) and Grunbaum, Kirk, Green, and Koch (1955).

Phosphorus—Schaffer, Frong, and Kirk (1953).

Fatty Acid—Schaffer, Grunbaum, and Kirk (1954).

As little as 0.2 to 1.0 mg. amounts of tissue or cells have previously been analyzed by these methods. (Grunbaum, Schaffer, and Kirk, 1955; Cailleau, Moss, and Siegel, 1956.)

Results

The reproducibility of the methods was tested on aliquots of a single, large sample of Ehrlich's mouse ascites tumor cells. These cells were easy to pipette and of relatively constant size. The uniformity of results obtained on replicates assayed at different times over a five-week period, is demonstrated in Table 1.

While results obtained with aliquots from a given source are remarkably constant, variation may be considerable, in material from different origins. As the source of the serum cannot always be identified adequately, especially in samples of pooled human serum, or commercially processed sera, wide variations are ob-

served. In tissue cultured cells, however, there appears to be a correlation between certain levels of the chemical constituents of the serum and its growth-promoting or toxic properties for the cells.

TABLE 1

Fatty acid and lipid phosphorus content of aliquots of Ehrlich's Mouse ascites tumor.

Date Assayed	Fatty acid μmoles/mg. N.	Lipid P μmoles/mg. N.	Fatty acid/ Lipid P ratio
Nov. 22	2.01	1.09	1.84
Nov. 23	2.06	1.09	1.89
Nov. 24	2.06	1.14	1.81
Nov. 29	2.09	1.12	1.87
Nov. 30	2.03	1.11	1.82
Dec. 3	1.97	1.02	1.93
Dec. 8	1.97	1.02	1.93
Dec. 30	2.17	1.02	2.13
Mean	2.05	1.08	1.91
δ	.06	.05	.10

Cord serum was found to be an excellent, uniformly non-toxic component of the growth medium, while pooled human serum was quite unpredictable, exhibiting extreme variability from batch to batch.

In examining the results in Table 2, certain trends are apparent. Fatty acid values which range above .40 are most frequently found in sera which are toxic or have poor growth-promoting properties.

Sera from known adult females (human, horse, rabbit and chicken), despite normal fatty acid values in some cases, were inadequate or toxic.

With the exception of rat serum, high fatty acid/lipid P ratios of 3.0 or more seem to coincide with poor growth qualities of the serum. Rat serum, tested on Earle's L cells, produced rather slow growth, with extremely granular cells, during the earlier subcultures, and only after seven or more transfers did the cells appear nearly normal.

Fowl sera show consistently low nitrogen and relatively high fatty acid values in all animals tested. Extremely high fatty acid values appear in hen serum, even after the animal has been fasted 48 hours.

TABLE 2

Nitrogen, Fatty acid, and Lipid Phosphorus composition of sera used in growth media in tissue cultures

Serum		Description	Chemical Composition of Sera				Growth of Cells
			Nitrogen mg./ml.	Fatty acid μmoles/mg.N	Lipid P μmoles/mg.N	Ratio of Fatty acid/Lipid P	
Cord	A	Pooled from 3	7.2*	.356	.196	1.82	Good
	B	or more indi-	7.3*	.346	.189	1.86	Good
	C	viduals	8.1*	.350	.150	2.35	Good
Human	No. 37	Pooled from 8	11.3	.397	.192	2.03	Good
	44	or more indi-	9.4	.504	.260	1.94	Poor
	45	viduals	10.0	.600	.310	1.93	Poor
	46	Selas filtered	9.9	.564	.284	1.98	Fair
	48		10.7	.500	.208	2.40	Poor
	R.C.	♀-fasted; Selas filtered	10.4	.550	.270	2.03	Poor
	Cutter	Pooled	9.9*	.418	.210	1.98	Fair
Monkey	(rhesus)	Pooled (2) filtered	9.4*	.424	.223	1.86	Poor
Horse	(Cutter No. 2956)	Pooled-4 filtered	10.7	.308	.135	2.22	Good
Horse	(Parker)	Pooled-fasted filtered	10.6	.236	.118	2.00	Good
Horse	(Pregnant ♀ mare)	Filtered	9.6	.274	.137	1.99	Poor

♀ mare)		Filtered	9.6	.274	.137	1.99	Poor
Ox	Cutter	Pooled 2 filtered	11.8	.178	.056	2.93	Fair
	Lee	1-filtered	11.1*	.210	.110	1.90	Good
Hog	A	Pooled 2 filtered	9.4*	.181	.062	2.83	Fair
	B	Pooled 2 filtered	12.0*	.220	.043	5.10	Poor
Rabbit	Cutter	Pooled 4 or more	7.5*	.350	.129	2.73	Poor
	Lee	Pooled ♀	7.7*	.337	.059	6.00	Poor
Lamb	fasted	2 or more	8.0*	.208	.105	1.98	Good
Ram	fasted	2 or more	8.6*	.136	.059	2.30	Good
Ewe	fasted	2 or more	6.8*	.145	.050	2.86	Good
Rat ♂ young (6 wks.)	fasted	Pooled 4 or more	8.6	.120	.030	4.00	Good but granular
Chicken: Hen fasted 48 hours		3-pooled	5.0	2.90	1.40	2.07	Poor (toxic ?)
Cockerel		2-3 pooled	7.2	.560	.262	2.13	Fair
Young-fasted chick (Cutter)		Pooled	6.1	.425	.190	2.28	Poor (toxic)

* 5 per cent solution containing penicillin and streptomycin added to serum before assay.

High fatty acid/lipid P ratios usually are indicative of a low lipid P content rather than a rise in fatty acids. When this occurs the serum is not necessarily inhibitory, but the cells which grow may appear dark, granular or abnormal.

The effects of different sera or media upon the lipid composition of Earle's strain L cells is illustrated in Table 3. Variations

TABLE 3

Fatty acid and lipid phosphorus content of Earle's strain L cells grown on various media

Medium*	Growth period	Chemical Composition		
		Fatty acid μmoles/ mg. N	Lipid P μmoles/ mg. N	Fatty acid/P ratio
40 per cent Horse	8 d.	2.64	1.56	1.69
serum No. 2272	13 d.	2.12	1.18	1.80
40 per cent Horse	9 d.	2.88	1.27	2.26
serum No. 2956	14 d.	3.40	1.37	2.48
30 per cent Horse	10 d.	2.84	1.54	1.85
serum No. 3046	13 d.	2.60	1.65	1.52
30 per cent Horse	15 d.	2.34	1.58	1.48
serum (HOK)	15 d.	2.17	1.33	1.63
30 per cent Ox serum....	14 d.	2.77	1.47	1.86
No. 17740	6 d.	3.33	1.57	2.12
30 per cent Ox	7 d.?	2.75	1.48	1.86
serum (Lee)		3.50	1.64	2.14
40 per cent Human	6 d.(§)	3.96	1.59	2.48
serum	6 d.(‖)	4.88	1.94	2.52
40 per cent Hog serum	32 d.	6.70	2.69	2.48†
40 per cent Rat serum	13 d.	2.96	1.59	1.86
40 per cent Ascitic ..... fluid	8–15 d.	4.00	1.81	2.21‡

* Serum, plus 40 per cent 199, 5 per cent solution of penicillin and streptomycin, and Tyrode's to complete to volume.
†Fifth transfer, growth slow and cells extremely granular.
‡Average value from 10 series of experiments using several different ascitic fluids.
§First transfer only.
‖Second transfer only; no growth obtained after third transfer.

which occur seem to reflect more the serum used, than the length of time in culture, and are most apparent in the fatty acid values. High fatty acid-lipid P ratios around 2.5 are found in cells grown on human and hog serum. Only three subcultures could be obtained in the experiments with human serum, and while good growth was obtained in the first two transfers which were as-

sayed, the cells were abnormal, amoeboid and granular. Similarly, the cells on hog serum grew very slowly at first and produced many granular and multinucleate forms.

A comparison of three types of cells on various media is shown in Table 4. HeLa cells which grow equally well on horse, ox and

TABLE 4

The Effects of Different Sera upon the Fatty Acid and Lipid Phosphorus content of cells in Tissue Culture

Serum	HeLa			L cells			Liver Epithelium		
	F.A.*	Lipid P*	FA/P ratio	F.A.*	Lipid P*	FA/P ratio	F.A.*	Lipid P*	FA/P ratio
Horse	3.0	1.6	1.9	2.5	1.6	1.6	3.7	2.0	1.85
Ox	2.8	1.4	2.0	3.0	1.5	2.0	3.9	2.1	1.9
Human	3.0	1.6	1.9	4.4	1.8	2.7†			
Chick–hen ...	4.1	2.1	1.9†						
Chick (Cutter)	5.0	1.9	2.6†						
Cock–fasted ..	2.7	1.5	1.8						

*Fatty acid and lipid phosphorus values calculated as μmoles per milligram Nitrogen.
†Average of two transfers only. No growth obtained after the third transfer.

human sera show essentially the same composition on all three media. Growth on chick serum was uniformly slower, less abundant and the cells were heavily granular, abnormal and incapable of continued subculture in sera with high fatty acid values. Growth was proportionately heavier and less granular in serum from the fasted cock and subcultures were maintained for 5 transfers.

Similar results are observed with L cells. Low fatty acid values in horse serum media correspond to clear normal cells; slightly granular cells were obtained in ox serum while growth in human serum was limited to 2-3 subcultures and was characteristically granular and abnormal.

Liver epithelial cells showed essentially the same appearance and chemical composition on horse and ox sera and seem to contain a somewhat greater amount of both fatty acid and lipid P when growing normally.

That this may be a normal function in liver tissue is perhaps illustrated in Table 5. In both fresh human embryonic tissue and cells grown for several months and subcultured many times, liver tissue seems to contain greater amounts of fatty acid than most of the other tissues. In the assays of fresh material the brain tissue contained the most fatty acid and lipid P, as was to be expected.

Lung and muscle were uniformly low in both components while the fatty acid/lipid P ratios for all four tissues were practically identical.

TABLE 5

Fatty Acid and Lipid Phosphorus Content of Some Fresh and Cultured Embryonic Human Tissues

Tissue		Chemical Composition Nitrogen mg./ sample	Fatty Acid µmole/ mg. N	Lipid P, µmole/ mg. N	F.A./ P ratio	
Brain		26.1	3.04	1.98	1.53	
	(fresh material	21.3	3.52	2.33	1.54	
Liver	from a 2½	33.3	2.27	1.45	1.59	
Lung	month old	45.3	1.59	0.97	1.65	
	embryo)	45.3	1.58	1.00	1.58	
Muscle		43.4	1.60	1.16	1.51	
	sub-culture					type cells
Muscle	11	72.0	2.63	1.73	1.52	fibro.
Liver	11	75.0	2.78	1.44	1.93	mixed
	21	26.6	3.09	1.62	1.91	fibro.
Lung	13	72.4	2.09	1.47	1.42	mixed
	22	82.4	2.50	1.26	1.98	fibro.
	23	41.8	2.86	1.43	2.00	fibro.
Cartilage	6	45.6	2.75	1.35	2.03	fibro.
	11	74.3	2.59	1.31	1.98	fibro.
	24	50.5	2.19	1.42	1.54	fibro.
Small Intestine	12	70.7	2.34	1.24	1.87	mixed-epith.
	20	97.0	2.22	1.16	1.91	
Skin	23	34.0	1.66	0.95	1.75	fibro.
Placenta	22	71.5	2.67	1.55	1.72	mixed

All cultures were grown in media containing 40 per cent cord serum, 8 per cent EE_{50}, 47 per cent Tyrode's and 5 per cent penicillin and streptomycin solution.

The cultured tissues tended to reflect the values of the serum more closely, while showing some changes after prolonged cultivation. Skin cultures gave extremely low values for both fatty acid and lipid P after 23 subcultures, and the fibroblast-like cells present were particularly clear and non-granular.

The effects of prolonged culture of spinal cord cells in human

and cord serum are shown in Table 6. The results seem to indicate that higher fatty acid values were predominant. Later subcultures run in parallel for the same length of time, to compare cord serum to human serum, show that those cells grown in cord serum contain significantly lower amounts of fatty acid.

TABLE 6

Fatty Acid and Lipid Phosphorus Content of cells from Human Embryonic spinal cord cultivated in vitro

Sub-culture	Days in culture	Serum	Chemical Composition			
			Nitrogen mg./ sample	Fatty Acid μmoles/ mg. N	Lipid P μmoles/ mg. N	FA/P ratio
7th	13	Human No. 37	44.4	6.73	3.0	2.24
8th	10	Human No. 37	27.9	6.31	1.47	4.32
17th	8	Human No. 45	8.6*	6.66	2.05	3.26
18th	7	Human No. 46	33.6	4.28	2.54	1.69
18th	7	Cord	31.5	3.66	1.57	2.29
19th	13	Human No. 46	30.3	4.05	2.25	1.82
19th	13	Cord	23.6	2.69	1.61	1.67
20th	12	Cord	30.6	2.39	1.68	1.42

*Small sample.

Discussion

It is difficult to draw any categorical conclusions when one deals with complex biological materials. Cells from various species react differently to homologous or heterologous sera. The duration of culture, number and type of cells, components of the media as well as components of the serum itself affect the appearance and growth of the cells.

Our values for serum nitrogen are generally in the range of those listed in Albritton's Standard Values in Blood (1952), however, apparent discrepancies might be attributed to the source or the method of preparing the serum.

Insufficient data on age, sex, fasting state, disease, etc., of the subjects providing pooled sera from human or other sources may be responsible for masking many factors which could influence the adequacy or toxicity of a serum.

While many other chemical components of serum and cells undoubtedly are interdependent and of primary importance in the growth of the cells, extreme values in fatty acid and lipid P fractions of serum seem to correlate with lower growth-pro-

moting properties or toxicity. The tissue cultured cells react to these unfavorable sera by showing increased granularity, rounded and abnormal forms, and in extreme cases crenation or total lack of growth.

Summary and Conclusions

A variety of sera was used in culture media for HeLa, Earle's strain L and liver epithelial cells. Microchemical analyses of the nitrogen, fatty acid and lipid P contents of both sera and cultures were compared. Fresh human embryonic tissues and subcultures from the same tissues were grown in cord or human sera and assayed for these same components.

The results indicate that the fatty acid and lipid P content of sera varies considerably from animal to animal and from species to species.

The ratio of fatty acid/lipid P is usually quite constant around 2:0. Higher ratios are usually caused by low lipid P values.

Sera showing fatty acid values of 0.4 μmoles per mg. of nitrogen have frequently been unsatisfactory or toxic for the cells. Cells grown in such sera become granular, enlarged, amoeboid or multinucleated.

High fatty acid values in serum usually induce an increase in the fatty acid content of the cells.

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* Department of Biochemistry and Virus Laboratory, University of California, Berkeley, California. Supported by grants from the U. S. Public Health Service, National Institutes of Health and the cancer research funds of the University of California to Dr. P. L. Kirk.

† Present address Cancer Research Institute, University of California Medical Center, San Francisco 22, California.

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RESOLUTION OF CONFLICTING DATA CONCERNING THE EFFECTS OF L-EPINEPHRINE AND L-NOREPINEPHRINE ON BLOOD PRESSURE AND HEART RATE OF UNANESTHETIZED DOGS*

RUVEN GREENBERG,† AND CHARLES B. LAMBETH‡

In a previous work (7) a graded series of intravenous doses of l-epinephrine and l-norepinephrine were administered by quick injection and by infusion to unanesthetized dogs, whose hearts had been previously denervated. Infusions of near-minimal doses of l-epinephrine caused vasodepressor and negative chronotropic effects; with larger doses both these effects were reversed. In contrast, l-norepinephrine invariably affected increases in heart rate and blood pressure. Similar data on intact unanesthetized dogs are presented herewith. We were particularly interested to learn whether near-minimal infusions of l-epinephrine would cause simultaneous vasodepression and bradycardia.

In several reports on the intact unanesthetized man (5, 1, 8), the pattern of effects on heart rate by these agents was reversed as compared to the above: at near-minimal doses l-epinephrine affected reflex cardio-acceleration and l-norepinephrine affected reflex slowing. In the only previously reported infusions of these agents into intact unanesthetized dogs, Gazes *et al.* (3) obtained still another set of data. They reported qualitatively similar effects: the blood pressure was elevated and the heart rate was slowed.

Our data, over a much wider range of doses than those used by the above authors, provides the explanation which resolves the above contradictory sets of data. Infusions of near-minimal effective doses of l-epinephrine and l-norepinephrine resulted in qualitatively different changes in blood pressure and heart rate whereas at the higher doses the effects of these agents became qualitatively alike.

In addition, a graded series of intravenous doses of l-epinephrine and l-norepinephrine were injected quickly into unanesthetized dogs. The resultant effects on blood pressure and heart rate are reported and discussed. We noted, especially, that there

was obtained a bipeaked arterial record (Fig. I) with l-epinephrine only and none with l-norepinephrine. The only other similar reference available to us was by De. V. Cotten and Pincus (2). Their data on blood pressure and heart rate for these experiments was fragmentary and indefinite and only roughly comparable to ours.

Methods

The methods used were similar to those described in previous papers (6, 7). 19 adult mongrel dogs (9–11 kgs.) were chosen on the basis of their cooperativeness. They were trained to lie quietly, without anesthesia, while fastened in a prone position. An indwelling arterial needle (heparinized) was inserted into a femoral artery. An indwelling nylon catheter was inserted for a distance of 20–25 cms. into a femoral vein. Continuous arterial tracings were made using a Statham Pressure transducer model No. P23A, amplifier, and ink recording.

1 mcg., 2 mcg., 10 mcg., and 100 mcg. doses of the bitartrate salts of aqueous l-epinephrine[1] and l-norepinephrine[2] in 1 ml. volumes were injected quickly (within 1 sec.) into the femoral vein catheter which was flushed immediately with a 1 ml. volume of physiological saline.

The same agents were infused under positive pressure at the rate of 1.2 mcg./min., 2.4 mcg./min. and 12 mcg./min. for the first three minutes, and at three times this dose at the rate of 3.6 mcg./min., 7.2 mcg./min. and 36 mcg./min. during the subsequent two minutes. The dosage for the infusions at the end of 5 minutes totalled 10.8 mcg., 32.4 mcg., and 108 mcg. respectively.

The solutions were freshly prepared and used within a few minutes after dilution. The usual time interval between injections was five minutes. Between 8–10 quick injections and 3 infusions (as described above) were given during one experiment. The order of the quick injections was from the small to the large doses. These were followed by the infusions. Placebo doses of physiological saline were administered from time to time. Total time for a given experiment varied from 60–90 minutes. This necessitated omitting some of the test doses in each experiment.

By and large the blood pressure and heart rate values returned to resting level within the five minute intervals between the injections except after the 100 mcg. doses which tended to excite the dogs. This necessitated longer intervals of rest after these doses; and on some occasions this necessitated repeating the experiment at a later date.

Thirty-six experiments were done on 19 dogs: 4 experiments on each of 3 dogs, 3 experiments on each of 4 dogs, and 1 experiment on each of 12 dogs.

Results

A. *Quick Injections*

Table 1 is a summary of the maximal changes of heart rate

TABLE 1

Comparison of maximal percentage change in heart rate (during 15 second interval) with quick intravenous injections of L-epinephrine and L-norepinephrine administered singly and in equal mixtures

L-epinephrine					L-norepinephrine					Mixture L-epinephrine + L-norepinephrine				
Dose	Mean Per Cent Rate Change		n*	d*	Dose	Mean Per Cent Rate Change		n	d	Dose	Mean Per Cent Rate Change		n	d
1 mcg.	+6	—	4	4	1 mcg.	—15	—	4	4	2 mcg.	—7	—	4	4
2 mcg.	+10	±23.2*	23	16	2 mcg.	—16	±9.5	19	13	4 mcg.	—15	±9.3	6	5
10 mcg.	—14	±26.0	25	18	10 mcg.	—32	±7.4	20	14	10 mcg.	—24	±13.2	14	9
										20 mcg.	—25	±12.3	11	9
100 mcg.	—33	±10.7	5	5	100 mcg.	—38	±10.7	6	5	100 mcg.	—44	±13.6	5	5
										200 mcg.	—48	—	2	2

n*Number of tests. d*Number of dogs. *Standard deviation.

during a 15 second interval in the first minute after quick intravenous injections. The mean per cent rate changes above or below the resting rate are indicated.

The quick intravenous injections of small doses (2 mcg., 10 mcg.) of l-epinephrine resulted not infrequently in bipeaked blood pressure curves (Fig. I). In the interval (10–20 seconds) between the blood pressure peaks there was recorded a well defined cardio-acceleration. The per cent rate increase thus obtained with these doses is listed in Table 1. L-epinephrine affected cardio-acceleration in 11 of 23 tests at the 2 mcg. dose and in 2 of 25 tests at the 10 mcg. dose, whereas only insignificant rate changes (± 6 per cent) were noted in 4 of 4 tests at 1 mcg., in 7 of 23 tests at 2 mcg., and in 2 of 25 tests at 10 mcg.

The bipeaked blood pressure curve was not obtained in the remaining tests at the lower doses; and not at all at the 100 mcg. dose; further it was not obtained either with l-norepinephrine or with equal mixtures of the two agents. Instead, at all these doses there resulted a simple pressor curve associated usually with an increased pulse pressure and a slowed heart. In only 2 of 19 of the 2 mcg. doses of l-norepinephrine and in 2 of 14 of the 10 mcg. doses made up of equal mixtures the heart rate slowing was insignificant (±6 per cent). In no case did the l-norepinephrine injections affect a cardio-acceleration.

If the blood pressure and heart rate values of the l-norepinephrine component of the equal mixtures were used as a baseline, then the effect of the added l-epinephrine was unpredictable. In a given experiment the degree of slowing in heart rate and increase in blood pressure might be unchanged, increased, or decreased. In no case was the simple pressor curve converted into a bipeaked pressor curve.

B. *Infusions*

The infusions of l-epinephrine (Table 2) at the lower doses caused a maintained decrease in blood pressure associated with a maintained increase in heart rate in 23 of 28 tests at the 2.4 mcg./min. level; and in 24 of 28 tests at the 7.2 mcg./min. level. There resulted an increase in blood pressure associated with a decrease of heart rate in 0 of 28 infusions at 2.4 mcg./min., and in 3 of 28 infusions at 7.2 mcg./min.

The infusion of l-epinephrine at the higher doses affected a

maintained increase in blood pressure associated with a decrease in heart rate. However, in 1 of 13 infusions at 12 mcg./min. and in 1 of 13 infusions at 36 mcg./min. there was effected a slight vasodepression associated with a marked cardio-acceleration.

The infusions of l-norepinephrine affected a maintained increase in blood pressure associated with a maintained decrease in heart rate in every instance.

TABLE 2

Comparison of maximal percentage change in heart rate with intravenous infusions of L-epinephrine and L-norepinephrine administered singly and in equal mixtures

Dose mcg./min.	L-epinephrine Mean Per Cent Rate Change		n*	d*	L-norepinephrine Mean Per Cent Rate Change		n	d	Mixture L-epinephrine + L-norepinephrine Mean Per Cent Rate Change		n	d
1.2	—	—	—	—	—26	—	3	3	—	—	—	—
2.4	+22	±24.2*	28	19	—21	±8.6	22	15	—6	±8.4	13	12
3.6	—	—	—	—	—39	—	3	3	—	—	—	—
7.2	+27	±29.3	28	18	—30	±12.4	22	15	—8	±11.1	13	13
12	—9	±19.1	13	10	—28	±8.2	9	7	—21	±13.6	7	5
36	—10	±14.8	13	9	—33	±9.0	8	6	—24	±13.6	8	6

n*Number of tests. d*Number of dogs. *Standard Deviation.

The infusions of equal mixtures of the two agents caused a maintained increase in blood pressure associated with a decrease in heart rate in 34 of 42 infusions at all the dose levels. However, in 2 of 13 infusions at the 2.4 mcg./min. and in 3 of 13 infusions at 7.2 mcg./min. there were slight changes in blood pressure associated with moderate cardio-acceleration.

If the blood pressure and heart rate values of the l-norepinephrine component of the infusion mixture were used as a baseline, then the added l-epinephrine was antagonistic. There resulted a compensation of effects. In several instances (see above paragraph) the increase in blood pressure and slowing in heart rate were converted into decrease in blood pressure and cardio-acceleration. In 3 infusions (once each at 2.4 mcg./min., at 7.2 mcg./min., and 12 mcg./min.) the blood pressure and heart rate were unaffected (less than ±6 per cent rate change).

Discussion

A. *Resolution of conflicting data*

Several authors (5, 1, 8) have infused l-epinephrine and l-norepinephrine into unanesthetized humans at levels sufficient

for significant cardiovascular responses. They all reported qualitatively different cardiovascular responses: The l-epinephrine caused an overall decrease in blood pressure and cardio-acceleration. The l-norepinephrine caused an overall increase in blood pressure and bradycardia. On a weight basis the above authors used a range of doses from .05–.70 mcg./kg./min.: however, most of the infusions were within the .1–.3 mcg./kg./min. dose range.

In intact dogs, with the infusions calculated on a weight basis as for the above authors, we obtained similar responses within the same dose range (.24 mcg./kg./min., .72 mcg./kg./min.). However, our dose range extended considerably higher to 3.6 mcg./kg./min.; and we alone report that with the higher doses this qualitative difference is lost as both these agents then effect only overall increase in blood pressure and bradycardia.

Upon comparison of infusions of the two agents, Gazes *et al.* (3) also obtained qualitatively alike cardiovascular effects in intact dogs. Similarly the dosage level was high (1 mcg./kg./min.). Unfortunately, Gazes *et al.* (3) did not report data for infusions at the lower doses.

The infusions of equal mixtures of l-epinephrine and l-norepinephrine in the dogs herein reported resulted in a neutralization of the cardiovascular effects just as Goldenberg *et al.* (5) had reported in man. However, since in the dogs whose heart had been previously denervated l-epinephrine did not antagonize the cardiovascular effects of l-norepinephrine (7) it is clear that this antagonism is somehow dependent upon the interplay between the buffer reflex mechanism and the direct effects of the agents. Further, since this antagonism is best shown with the infusions and not with the quick injections of these mixtures, then probably it is dependent upon the maintained vasodepressor activity of the l-epinephrine component.

As in the dogs whose hearts had been previously denervated (7) the infusions showed best the vasodepressor effects of l-epinephrine. Further, any direct negative chronotropic activity such as was commonly shown with the l-epinephrine infusions in the dogs whose hearts had been previously denervated (7), was masked probably by reflex tachycardia. However, in 2 of a total of 82 infusions of l-epinephrine throughout the entire dose range there was noted a slowing in heart rate not associated with an increase in blood pressure.

B. *Genesis of bipeaked arterial record*

Bipeaked arterial records were made with the quick injections of l-epinephrine only. They were not obtained with any of the infusions. They were most frequently obtained with the near-minimal effective doses of l-epinephrine (2 mcg.). They were not obtained either with the l-norepinephrine or with the equal mixtures of the two agents. They were also obtained with near-minimal effective doses of commercial adrenaline[3] (Fig. I) in which l-epinephrine is the principal component (6).

FIG. I. Bi-peaked arterial tracing following i.v. quick injection at arrow mark of 2 mcg. of epinephrine in intact unanesthetized dog. The heart rates are indicated for 15 second intervals. Systolic and diastolic blood pressures are indicated at various points.

In previous papers from our laboratory, the quick injections of the same agents (7) and of commercial adrenaline (6) to dogs with denervated hearts resulted only in simple pressor or simple depressor curves. It would seem therefore that the genesis of this bipeaked curve was dependent upon reflex mechanisms; and further, it would seem that only l-epinephrine and neither l-norepinephrine nor a mixture of these agents were necessary for this effect. Probably this is explained by the comparative efficacy of the separate agents as mediators to the presso-receptors. L-epinephrine causes simultaneous vasodepressor and vasopressor effects whereas l-norepinephrine is primarily a vasopressor agent. Thus, l-norepinephrine causes a maintained state of increased tone and tension of barosensitive arterial wall for maintained reflex slowing of the heart rate. Consequently, 1-norepinephrine does not cause a bipeaked curve.

Most of the changes in heart rate that resulted, with both the quick injections and the infusions, were in accordance with Marey's reflex. However, in one instance (1 of 148) a 2 mcg. quick injection of l-epinephrine effected a significant increase in blood pressure not associated with a bradycardia but rather with a 20 per cent cardio-acceleration, and in 11 of 190 infusions a

similar result was effected. Probably in these exceptional cases the direct cardio-acceleration by the l-epinephrine overshadowed the reflex slowing usually mediated via the buffer nerve mechanism while the arterial blood pressure is increased. Such exceptional cases were never noted with the l-norepinephrine infusions; and only rarely with the infusion of the equal mixtures.

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Summary

Intravenous quick injections and intravenous infusions of l-epinephrine, l-norepinephrine, and equal mixtures of these agents were administered to intact unanesthetized dogs through a wide range of doses.

1. The intravenous quick injection of small doses of l-epinephrine only resulted in bipeaked pressor curves. By comparison, intravenous quick injections of l-norepinephrine affected simple pressor curves associated with bradycardia.

2. The intravenous infusion of small doses of l-epinephrine resulted in a well maintained decrease in blood pressure associated with reflex tachycardia. By comparison, intravenous infusions of l-norepinephrine resulted in a well maintained increase in blood pressure associated with reflex bradycardia.

3. The infusions, as compared with the quick injections, best showed the vasodepressor effects of l-epinephrine and thus elucidated the observed antagonism between l-epinephrine and l-norepinephrine seen best with the infusions of equal mixtures.

4. The data herein reported were discussed in comparison with similar data in man and in comparison with similar data in the unanesthetized dog whose heart had been previously denervated.

NOTES AND PAPERS CITED

* From the Department of Physiology, University of Illinois College of Medicine, 1853 West Polk Street, Chicago, Illinois and The Carter Physiology Laboratory, The University of Texas Medical Branch, Galveston, Texas. Received for publication January 1, 1957.

† Present Address. University of Illinois College of Medicine, 1853 West Polk Street, Chicago 12, Illinois.

‡ Present Address. Box 1107, Kingsville, Texas.

[1] L-epinephrine as epinephrine bitartrate (R-041) kindly supplied by Dr. M. L. Tainter of the Sterling Winthrop Research Institute.

[2] L-norepinephrine as 'Levophed Bitartrate Solution 0.2 per cent' manufactured by Winthrop-Stearns, Inc., New York, New York.

[3] Commercial epinephrine. 3/200 grain tablets. Parke Davis and Company (approximate composition: l-epinephrine 82 per cent, l-norepinephrine 18 per cent). (4)

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DEVELOPMENT OF PEDIATRICS AT THE UNIVERSITY OF TEXAS SCHOOL OF MEDICINE IN GALVESTON*

ARILD E. HANSEN AND MARY ELLEN HAGGARD

In reviewing the development of activities related to Pediatrics at The University of Texas School of Medicine in Galveston, it appears pertinent to refer to some historic highlights related to this institution. Although the Constitution of Coahuila and Texas (1827) provided for public education, (1) realization that education was the basis of a sound democracy became strikingly evident in one of the first public expressions concerning Texas. One of the charges set forth in the Declaration of Independence of the Republic of Texas on March 2, 1836 reads: Mexico "has failed to establish any public system of education, although possessed of almost boundless resources (the public domain), and although it is an axiom in political science, that unless a people are educated and enlightened, it is idle to expect the continuance of civil liberty, or the capacity of self-government." (2) It was written into the Constitution of the Republic of Texas (March 17, 1836) that there be a general system of education. (2)

The University of Texas

It was only nineteen months after the Battle of San Jacinto (April 21, 1836) that General Kelsey H. Douglass introduced in the Second Congress, Regular Session, of the Republic a bill to incorporate The University of Texas (1837). In the Adjourned Session of the Second Congress (1838) this bill was referred to a select committee. (2) In 1839, there were set aside 50 leagues of land as an endowment for two colleges or universities. (3) The Constitution of the State of Texas (1845), Article Ten, provided for state support and maintenance of public education. (4) The messages of succeeding Governors to the Legislatures pleaded for the State University and bills were introduced at the various legislative sessions. In the ensuing years much discussion took place concerning the creation of the university and some comments were not favorable. On November 24, 1857 at the House

of Representatives' meeting in discussing a report of the Committee on Education in relation to the creation of one or more State Universities it was stated, "Let those who may wish to receive what is called the genteel polish of a 'finished professional education,' not call upon the people in the humble walks of life, upon whom they may in future show their professional skill, in fleecing or physicing, to pay for educating them. Let us have no Doctors or Lawyers manufactured by a State institution; and I say it with all becoming respect to gentlemen of the learned professions, God knows we have enough of them in the country, with a rising prospect of 'more of the same sort!' " The predominating opinion, however, favored the creation of a state university, and in 1858 a legislative act to establish a university was passed. The act gave $100,000 of the United States Bonds in the Treasury; 50 leagues of land; and one section of land to every 10 granted to railroads. Among the subjects specified to be provided for were "Anatomy, Surgery and Medicine." (6) In 1860 the $100,000 in U. S. Bonds set apart to the university was enacted to be used for frontier defense. (7) No doubt because of war, nothing was done to start the university; however, considerable discussion of the subject took place. That there should be a university of the "first class" to be located by the people was written in the Constitution as Article VII in 1876. (8) Without doubt, inasmuch as by this time the railroads had been given 30 million acres, even to the Texians, the 3 million acres began to appear quite sizeable, hence the Constitution of Texas gave 1,000,000 acres for the university in lieu of the 1 to 10 ratio for the railroads. (9) It was in 1881 that the act to establish the university was passed with the location to be determined by public referendum. "The medical department of the university shall be located, if so determined by a vote of the people at a different point from the university proper and as a branch thereof; . . ." (10) In the message of Governor O. M. Roberts to the Eighteenth Legislature, Regular Session, on January 10, 1883, he stated that "this University of Texas, its main branch, its medical branch, and branch for colored youths have been located by vote of the people." (11) The ballot was reported by the Secretary of State and Governor as follows: for Austin for the Main University 16,306 votes, for entire University, 14,609 votes, total for Austin 30,913 ovtes; (The corresponding figures for Tyler were 18,420, 554, 18974; for Waco

2,632, 7,167, 9,799; the total remaining scattered vote was less than 7,000.) for Galveston for the location of the Medical Department 29,741; Houston 12,586; Austin 994; scattering 350. (12) During the next few years there was considerable discussion concerning university lands, administration and available funds. During the proceedings of the Twentieth Legislature, Called Session, attention was given to the Medical Branch. As a loan to the available fund of The University of Texas, credit to the extent of $125,000 was given of which sum $50,000 was to be used for buildings for the Medical Branch at the city of Galveston. "*Provided*, that the said City of Galveston shall donate to The University of Texas, Block No. 668 in said city, to be used for the Medical Branch of said institution; *and provided, further*, That the executors of the estate of John Sealy, deceased, shall agree to construct on said block, at a cost of not less than $50,000, a medical hospital, which, when completed, is to be donated to the Medical Branch of The University of Texas, and to be under the control of the Board of Regents. . . ." (13)

Medical Schools

In 1860 the first medical school of Texas was established in Galveston. This was the medical department of Soule University and was known as Galveston Medical College. Soule University was founded in 1856 at Chappel Hill, Washington County, by the Texas Conference of Methodist Episcopal Church, but according to the records, the Medical College did not function. In 1866 the Galveston Medical College was re-organized by Soule University; however, the Medical School closed when Soule University moved to Louisiana seven years later. Immediately, the "Texas Medical College and Hospital" was organized for which $5,000 had been appropriated annually. The professors were selected by competitive examination. Although the vote of the people of Texas chose Galveston as the site of the Medical Branch of The University of Texas, no definitive activity was taken in this direction until 1899, principally because of no support for a hospital. It was then proposed that the Texas Medical College and Hospital be re-organized and furnish the medical and surgical staff until the State Medical School could be organized. Among the nine professors chosen, only one received a salary, and the

entire budget was spent for his salary plus a laboratory. The period of study was for three years of five months each. At this time the Legislature appropriated $50,000 for the construction of the medical college building, although there was considerable question concerning the authority of the Legislature to appropriate the money. To help solve the establishment of a much disputed feature, namely a hospital, the City of Galveston transferred John Sealy Hospital to the Board of Regents of The University of Texas.

Teachers of Pediatrics

In 1866 William H. Boring, M.D., was listed on the first faculty of Galveston Medical College as Professor of Obstetrics and Diseases of Women and Children. He also served as a Methodist minister and was a much respected gentleman. The Island City Hospital had been leased for teaching purposes by Dr. Greensville Dowell, Professor of Anatomy and Dean of the Faculty. From the Galveston Medical Journal which was the first periodical of the Galveston Medical College, we learn that the institution did not prosper as hoped. The faculty resigned in body when Dr. Dowell refused to resign and for some time he filled all the chairs. In 1873, following withdrawal of support of Soule University, examinations of candidates for professorships at the Texas Medical College and Hospital were announced by Dr. Ashbel Smith, President of the Board. The announcements were published in three daily newspapers for 60 days, and invitations also were sent to the medical profession. Seven men were selected for professorships and we note that none were for children; hence, we may assume that no one was qualified to teach the care of children. In 1880 C. W. Truehart, M.D., was Professor of Clinical Diseases of Women and Children. The description of his course as given in the 1880–81 catalogue is "the diseases of infants and children and the peculiarities of organization and function incident to infancy and childhood, and the system of hygiene and therapeutics applicable to these, will be carefully investigated." (14) Two textbooks on *Diseases of Children* recommended were one by Meigs and Peppers and the other by J. Lewis Smith.

The first session of The University of Texas School of Medi-

cine at Galveston began October 5, 1891. There was a faculty of 12 men of whom Dr. Henry Pendleton Cooke was Lecturer on Diseases of Children. There were 23 students and the budget was $4,500. Later, Dr. Cooke became Dean of the School. In the 1903–04 catalogue William S. Carter, M.D. is listed as Lecturer on Pediatrics, also Professor of Physiology and Hygiene and Dean of Faculty. In 1904–05, Wallace Rouse, M.D. is given as Lecturer on Pediatrics, and two years later his other duties included Demonstrator of Clinical Medicine and Instructor of Physical Diagnosis. From 1908–12, James Greenwood, Jr., M.D. is listed as Lecturer in Pediatrics, Demonstrator of Clinical Medicine and Instructor of Physical Diagnosis. In 1912, Allen George Heard, M.D. had been named Adjunct Professor of Pediatrics and Instructor of Medicine, and in 1916–17 Associate Professor of Pediatrics and Medicine. During 1916–18 we find that William Boyd Reading, M.D., a graduate of the class of 1914, had been appointed as Instructor in Clinical Medicine "on leave." From 1917–19 William Gammon, M.D. is listed as Associate Professor of Pediatrics and Medicine. After the war, Dr. Reading became Adjunct Professor of Pediatrics, promoted to Associate Professor in 1923, and named Professor in 1924. (15)

Hospital Facilities for Children

The first hospital on Galveston Island is pictured in several places and it is not difficult to imagine what type of care may have been given to children in the building depicted. In 1866 the Island City Hospital was leased for teaching purposes. Later, this facility became known as the City Hospital. It has not been possible to learn the nature of the hospital facilities for children, but as was the situation generally, undoubtedly these were meager—a situation not unexpected in the light of the high death rates for infants and children during these times.

The development of hospital facilities in Galveston is epitomized in the name Sealy. (16) John Sealy was born in Kingston, Pennsylvania in 1822 and came to Texas in 1840. He settled in Galveston in 1846 and was in partnership with J. H. Hutchings in commission business until 1854. Upon his death in 1884 from the terms of his will there would be established a hospital. Recently, when the new John Sealy Hospital was dedicated, the

local newspaper carried an item which revealed that Mr. Sealy had had friends visiting him whose children had become very ill. It was necessary that they be taken to a medical center far distant from Texas and he believed that such a situation should not exist. During the trying days of the establishment of the medical department of The University of Texas, the great handicap had been the lack of hospital facilities suitable for the establishment of an integral part of a University of the "first class." A distinct step to correct this situation was taken when the executors of the John Sealy estate decreed that there be: a medical hospital at a cost of not less than $50,000 to be donated to The University of Texas under control of the Board of Regents, and the City of Galveston was to donate the land. The location of the original John Sealy Hospital is said to be at the same site as the City Hospital—formerly the Island City Hospital. Again, it has not been possible to ascertain just what was the nature of the facilities for children in the first John Sealy Hospital.

That definite interest concerning hospital facilities for children was extant in Texas is apparent in the fact that in 1912 the Anti-Tuberculosis Association of the State of Texas provided $13,000 to build a Children's Hospital. It perhaps was no accident that the Anti-Tuberculosis Association rallied to the support of children, especially when one realizes that Dr. William S. Carter, formerly Lecturer on Pediatrics and Dean of the Faculty had long been active in the tuberculosis problem and was President of the Association at the time. By an act approved February 28, 1915, the State accepted the gift by the Public Health Association of the Walter Colquitt Memorial Children's Ward of the John Sealy Hospital, on the premises of The University of Texas, Galveston, the same to be the State Hospital for Crippled and Deformed Children and to be under the control and management of the Board of Regents of the University, and said Board was authorized to lease the same to the City in the same manner as the John Sealy Hospital Building. (17) It was further provided that the Legislature should make suitable provision in the general appropriation bill, or otherwise, to pay for the proper care of children afflicted with surgical tuberculosis. Such Children's Hospital was constructed by the Public Health Association at the expense of $15,000.00. The care of the children was augmented by the Hospital Aid Society which spent about $2500 a year on

clothes and occasionally for some apparatus. This building was located to the West of the John Sealy Hospital. It was not long before it was realized that the facility so provided was inadequate. After considerable discussion and activity in 1936 the present Children's Hospital was built. This was financed by a contribution from Federal public funds matched by funds from the University at a cost of $216,000.00. The Sealy and Smith Foundation contributed to the cost of building and furnishing the new Negro Hospital and the new Children's Hospital the sum of $107,313.00. The old Children's Hospital was remodeled to provide 25 beds for contagious diseases.

A great deal is said regarding the endowment of The University of Texas and the creation of the "available fund." The question often is asked, "Why does not the University build suitable hospital facilities to meet the needs for teaching purposes?" Dudley K. Woodward, Jr., Chairman of the Board of Regents, in a speech at the Texas Railroad Lawyer Convention in Houston on October 10, 1949, stated "this income from the available fund is the only source of funds for building at the Constitutional Branches of the University at Austin and Galveston, and that, so long as building needs exist at either place, it should be applied to buildings rather than to operating expense." It so happened that the hospital facilities for children became such that the Board of Regents meeting in Galveston in the Spring of 1955, as an emergency measure appropriated $200,000.00 to be used for the remodeling of the Children's Hospital. It was stated at this time that the Board of Regents had "scraped the bottom of the barrel" of the available fund in order to improve the hospital facilities for children. Everyone interested in the University's role in regard to medical care of children with its great teaching responsibility was extremely grateful for this action. Although the John Sealy Hospital was not built to provide the special facilities needed for the care of children, it was extremely fortunate to have facilities made available on the ninth floor of the grand new structure. The long term plans at the Medical Branch called for the addition of 1 to 2 floors to the present Children's Hospital, however, it is a question as to whether this will ever come about. In a study made by the American Academy of Pediatrics wherein each and every medical school in the country was rated, The University of Texas

Medical Branch's weakest point was the number of hospital beds for children, especially in relation to the size of the student body. We believe that a forward stride has been made in improving these facilities, and it is hoped that eventually clinical facilities especially suitable for the care and management of the small infants and children, as well as children with emotional and psychiatric disturbances, will be provided to meet the needs for teaching at the School of Medicine.

Teaching and Research

The growth and development of Pediatrics at The University of Texas Medical Branch followed along the same lines as occurred in other parts of the United States. The first outcroppings occurred during the latter half of the nineteenth century. In 1880 the Section on Diseases of Children of the American Medical Association was organized and in 1888, the American Pediatric Society, which was one of the first specialty groups to form in the United States. For the most part the early pediatricians came from or received their specialty training in Europe. Abraham Jacobi is generally recognized as the father of American Pediatrics, and it was he who pointed out that pediatrics does not deal with miniature men and women with reduced doses and with the same class of diseases in smaller bodies, but it has its own range and horizon and gives as much to general medicine as it receives from it. By no means has the growth of the specialty been uniform throughout the country; however, distinct trends were discernible at the turn of the century. In some areas departments of pediatrics were created and children's hospitals were begun, whereas in others, the subject of diseases of children was not taught at all, or lectures were given by someone in the public health, obstetrics or general medicine departments. About the time of World War I, separate departments of pediatrics and appointments as professor of pediatrics were springing up throughout the country and coincidental with this move, many state pediatric societies were started as was the Section on Pediatrics of the Southern Medical Association.

Many were the struggles of the physicians pioneering in the field of pediatrics. Almost always there was skepticism and even antagonism when revolutionary procedures were instituted

such as: radical changes in the method and attitude toward feeding infants; registering signs of being appalled at the high infant death rates; taking action to improve child health such as administration of diphtheria toxoid; as well as the development of well baby conferences with emphasis on *prevention of disease.* During the last part of the nineteenth and early part of the twentieth centuries, bacterial counts of the milk supply in many cities often were little different than those of sewage. The nidus of pediatrics, of course, was learning to feed the baby and without doubt the saving of lives accomplished by the application of this art and science has surpassed the life saving value of any of our great medical discoveries. The influence of pioneers of pediatrics was very great and the growth of the specialty may be appreciated by the statement of Smith (18) who pointed out that in 1900 only 1 in 2,500 physicians limited themselves to pediatrics, whereas in 1940 it was about 1 in 60. The ratio is even greater today. The American Academy of Pediatrics was organized in 1930, and at present has some 5,000 members. The American Board of Pediatrics was established in 1933.

The growth of the Department of Pediatrics at The University of Texas School of Medicine at Galveston has paralleled that of the other clinical departments. Pediatrics as a separate department was created in 1924 with Dr. Reading as the Head (and for sometime almost the entire body too). In the 1925–26 catalogue we find Edith M. Bonnet, B.A., listed as tutor in pediatrics. During 1926 and 1927 Dr. Archie Lane Mitchell served as Instructor in Pediatrics. From 1928 to 1936 Dr. Reading had Dr. James R. McMurray, Instructor (Assistant Professor in 1936) to assist him. In 1937 Francis A. Garbade joined the staff. In 1942 the total faculty at the institution was 158 members including 14 residents and 11 interns. At this time Dr. Reading had to assist him one Associate Professor, one Assistant Professor, both of whom had been appointed on a full-time basis, and four instructors, one of whom had a full-time appointment. There was one Resident assigned to the pediatric service (15).

In 1942–43 the authorities of the University created The University of Texas Child Health Program and were fortunate enough to obtain a grant of $200,000.00 from the William Buchanan Foundation of Texarkana to carry on this effort. After considerable discussion among the administrative authorities and

the Chairman of the William Buchanan Foundation, it was agreed that the activities should be centered in that department of the University which has most to do with children, namely, the Department of Pediatrics. The grant was for a five year period with the only stipulation that it be used to improve the health of the children of Texas by stimulating teaching and investigative activities at the University level. In the summer of 1943 the senior author was asked to accept the position of Professor and Head of Pediatrics, Director of the Child Health Program, Medical Director of the Children's Hospital and Pediatrist-in-Chief of the John Sealy Hospital. After many conferences with Dr. Boyd Reading, the President and Vice-President of the University and Dr. Stanley J. Seeger, Chairman of the William Buchanan Foundation, it was agreed that the Program was to be a continuous effort integrated with the Department of Pediatrics and the faculty had agreed to a full-time appointment. The basic principals and objectives of the Child Health Program were published in 1944 (19) and a report of activities from 1944 to 1947 was made in the Texas State Journal of Medicine (20). Splendid cooperation was obtained with Dr. Reading and Dr. Garbade both of whom were busy with their wartime heavy practice and also with Dr. E. E. Wilkinson who was full-time Assistant Professor. The fine understanding and keen interest of Dr. Seeger, who for many years had been a pediatric surgeon and the Chief of Staff of the Children's Hospital of Milwaukee, was very helpful. Additional stimulation was obtained from Dr. Chauncey D. Leake who was intensely interested in a Health Program for Texas. The helpful and cordial guidance of fellow faculty members and the administration made it possible to effect a distinct expansion in the department. It was possible to add a number of physicians to the staff, open a premature nursery, develop new laboratory facilities including the erection of a building as an animal colony, hold periodic pediatric conferences for practicing physicians, and appoint chemists, bacteriologists and child psychologists to the staff. Because of an enthusiastic teaching staff and a dedicated corps of clinical and laboratory investigators, teaching and research readily became prime objectives.

The fields of investigative efforts of the staff members have pertained to a rather wide range of subjects. Considerable work has been done in the field of lipid metabolism, especially in ref-

erence to the essential fatty acids. Other subjects have included: value of penicillin for congenital syphilis; causes and treatment of infantile diarrhea; synergism between aminophyllin and ephedrine; thallitoxicosis; potassium deficiency; diet in relation to endocrine function; adrenogenital syndrome; non-cellular antigens in pertussis immunization; changes in the spinal fluid in poliomyelitis; biologic antagonists in leukemias; vitamin B_6 and infantile convulsions; mineral balance in osteogenesis imperfecta; hemoglobin in sickle cell disease; hormonal agents in rheumatic fever and nephrosis; etiologic factors and evaluation of intelligence in the cerebral palsied child; nature of immune reaction in *H. pertussis;* and the effect of antibiotics on the bacterial flora of the nasopharynx. Quite naturally, many of these investigations have been carried on conjointly with members of other departments of the university. Particularly pleasant have been the studies carried on with D. C. Darrow of Yale on the role of potassium in the treatment of dehydration of infants with diarrhea; L. Emmett Holt, Jr. of New York University on amino-acid requirements of infants; Edward L. Pratt of the Southwestern Branch of The University of Texas at Dallas on the significance of fat in the diet of the premature infant; and Sidney Kaliski and associates in San Antonio on the problem of infantile diarrhea. The assistance of the State Department of Health in creation of the unit for premature care and the development of the Post-graduate Courses in Pediatrics has been very helpful. The interest of many members of the Texas Pediatric Society has been inspiring. The initial support from the William Buchanan Foundation was immeasurable in many of these efforts. Liberal support for research activities has been obtained from such sources as: the National Live Stock and Meat Board; the United States Department of Agriculture, Bureau of Home Economics and Human Nutrition; Sharp and Dohme Laboratories, Inc.; Hoffmann-LaRoche, Inc.; Wyeth and Company; the Baker Laboratories, Inc.; and the United States Public Health Service.

The residency training program has been considered a function of major importance. Some sixty physicians have received all or part of their experience in the department. Although most of them have remained in Texas, some have gone to parts quite distant from the homeland. Before World War II there was but

one resident and one interne assigned to pediatrics at The University of Texas Hospitals, whereas at present there are twelve residents, and six internes on this service. Not surprising, the services of many of the resident physicians have been split because of a call to military service.

The staff as now constituted is comprised of four professors, one of whom is a chemist and nutritionist; three associate professors, one of whom serves on a part time basis and another as a child psychologist; seven assistant professors, two of whom are part time physicians, one a bacteriologist and another a child psychologist; five instructors, two of whom are part time physicians, and one a child psychologist; and two research assistants. In carrying on the activities in the Child Behavior Clinic there is the valuable assistance of two members of the staff of the Department of Psychiatry, and a psychiatric social worker. One additional social worker has been assigned to pediatrics by the Social Service Department.

The most significant function of the Department of Pediatrics is student teaching which responsibility has been particularly great since the size of classes have been increased. Before institution of the Child Health Program in 1943, there were 100 students in the entering class which number during the war time period dropped to 80 to 90 by the time of the senior year. During this interval the Junior and Senior medical students spent about 1/10 to 1/12 of their clerkship assignment on this service. This situation gradually has changed. The entering class is now 164 with about 150 following through to the senior year. The time allocated to pediatric clerkship period is 1/4 of the total clerkship assignment for the junior year and 1/6 for the senior year. The change in time allocated by the administration to pediatrics was brought about by an awareness of the fact that a large proportion of the practice of the general man was in the field of pediatrics. The correctness of the assumption was strikingly apparent in the study made by the American Academy of Pediatrics on Child Health Services in the United States. Three out of four children taken care of medically were found to be under the care of the general practitioner, (21) hence it seems obvious that the medical school is where most potential physicians acquire their pediatric knowledge and experience. Essential to achieve this end is intimate experience with the child and his family during illness.

In the report made by the American Academy of Pediatrics on the Study mentioned, our weakest point was hospital facilities for children. With the increase in the size of the student body, shortage of clinical facilities reached the critical stage. The service as now constituted has about 90 beds for infants and children which are about equally divided between surgical and medical cases; 32 newborn cribs and 14 for premature infants. The new case per student per week varies from 0.5 to 1.0 in contrast to an expected 2.5 to 3.0. In other words for proper treating the needed 150 beds for infants and children including those for the emotionally or psychiatrically disturbed would be almost too much to hope for; but in view of the progress which has been made, doubling the number now available to The University of Texas Medical Branch would assist us in protecting our Achilles' heel. The members of the staff value greatly the interest of the friends of the department and are trusting that their continued interest will help to achieve the goal of accomplishment in teaching and research expected and that the excellent cooperation of the other medical schools in our state, the Department of Health and the medical societies will continue.

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INCIDENCE AND INTENSITY OF HOOKWORM INFESTATION IN CERTAIN EAST TEXAS COUNTIES WITH COMPARISON OF TECHNICS*

HAROLD E. HENDERSON

The problem of hookworm infestation remains of sufficient import in certain areas of East Texas to warrant the consideration of those interested in Public Health. Since Stiles described the worm in 1902, there has been only one thorough survey in Texas, that of the Rockefeller Sanitary Commission in 1912–1914. Upton (1936) reported a small survey conducted in 1932–33 in five east Texas counties in which he gave intensity of infestation on only a few specimens. Scott (1945) gave a resume of hookworm infestation in Texas adding statistical data of two more recent surveys. In this communication, we present information on degree of infestation and intensity as obtained from fecal specimens examined over a five year period, 1948–53, in the laboratory of the Texas State Health Department.

Methods

The specimens were collected by private physicians, health officers, school nurses and in some instances by teachers in the rural schools. Small, metal ointment boxes were used for containers. Most specimens were shipped to the laboratory without refrigeration or preservative, reaching us in most instances 2–4 days after collection. Specimens were kept under refrigeration at approximately 3–4° C. until examined. Examinations were made 1–30 days after the specimens were received.

All specimens were examined by the zinc sulphate floatation technic. Approximately 0.5 gm. of feces was thoroughly emulsified in approximately 8 ml. of zinc sulphate solution of specific gravity of 1.180, covered with a glass slide (75 × 37 mm.) and allowed to remain a minimum of 30 minutes before examination. A simple direct smear was examined on approximately 90 per cent of the fecal specimens. Both these preparations were stained with iodine to facilitate identification of other parasites found. A modification of the small drop dilution egg-counting technic, using displacement as described by Stoll and Hausheer (1926), was performed on specimens which were found positive by either the concentration or simple direct smear, except in a small per cent due to insufficient specimen.

Results

Incidence. Out of 8308 specimens examined from eleven counties, 2771 or 33.4 per cent showed hookworm infestation,

table 1. The largest number examined from one county was 2622 from Jasper County which showed 874 or 33.3 per cent positive. The highest per cent positive, 51.3, was from Tyler County, the lowest, 19.2 per cent, in Polk County. Out of 406 specimens from negroes, only 7 or 1.7 per cent were positive, table 2.

TABLE 1

Incidence of Hookworm in East Texas
1912–1914 and 1948–1953

COUNTY	1912–1914* Number examined	Number positive	Per cent positive	1948–1953 Number examined	Number positive	Per cent positive
Jasper	1016	603	59.3	2622	874	33.3
Trinity				1497	451	30.1
San Jacinto	626	546	87.2	1142	425	37.2
Hardin	1114	669	60.6	655	199	30.4
Liberty	600	361	60.1	630	131	20.8
Montgomery	999	700	70.7	536	189	35.3
Newton				494	215	43.5
Tyler				351	180	51.3
Houston	706	388	54.9	156	40	25.6
Angelina	1096	820	74.8	152	53	34.9
Polk	861	487	56.5	73	14	19.2
Totals	7018	4574	65.2	8308	2771	33.4

*Data for early study taken from Third and Fourth Annual Reports, The Rockefeller Sanitary Commission for the Eradication of Hookworm Disease.

TABLE 2

Incidence of Hookworm in Colored People

County	Number Examined	Number Positive	Per cent Positive
Angelina	57	1	1.8
Trinity	145	0	0
Jasper	136	6	4.4
Polk	68	0	0
Total	406	7	1.7

Intensity. The findings were classified in accordance with the suggestion of Smillie and Augustine (1925). Egg-counts were performed on 2373 fecal specimens; 51.8 per cent were very light, 23.5 per cent were light, 17.1 per cent were moderate, 4.3 per cent were heavy and 3.3 per cent were very heavy infestations, table 3. The number of egg-counts performed by counties ranged from 582 in Liberty to only 35 in Houston. In eight of the eleven counties surveyed more than one hundred egg-counts were performed. Slightly over 75 per cent of the egg-counts were

TABLE 3

Intensity of Hookworm Infestations in East Texas

County	Total Specimens Egg-Count Performed	Very Light Number	Per cent	Light Number	Per cent	Moderate Number	Per cent	Heavy Number	Per cent	Very Heavy Number	Per cent
Liberty	582	323	55.5	136	23.4	91	15.6	11	1.9	21	3.6
Trinity	368	246	66.9	70	19.0	35	9.5	9	2.4	8	2.2
Jasper	267	171	64.0	57	21.3	33	12.4	4	1.5	2	0.8
Tyler	264	94	35.6	65	24.6	60	22.7	24	9.1	21	8.0
San Jacinto	245	107	43.7	76	31.0	45	18.4	11	4.5	6	2.4
Montgomery	236	99	41.9	59	25.0	54	22.9	15	6.4	9	3.8
Hardin	145	70	48.3	34	23.4	34	23.4	6	4.2	1	0.7
Newton	144	64	44.5	35	24.3	31	21.5	11	7.6	3	2.1
Angelina	44	21	47.7	7	15.9	12	27.3	3	6.8	1	2.3
Polk	43	12	27.9	12	27.9	6	14.0	7	16.2	6	14.0
Houston	35	21	60.0	6	17.1	5	14.3	2	5.7	1	2.9
TOTAL	2373	1228	51.8	557	23.5	406	17.1	103	4.3	79	3.3

in the very light or light intensity. In the moderate to very heavy, Trinity County showed the lowest with 14.1 per cent, and Polk, the highest with 44.2 per cent.

Comparison of efficiency of technics employed. The efficiency of the technics employed in this study is shown in table 4. Out of

TABLE 4

Comparison of Efficiency of Technics

	Very Light	Light	Moderate	Heavy	Very Heavy	Number	Per Cent
Positive Floatation Positive Direct Positive Egg-Count	172	185	204	53	47	661	53.9
Positive Floatation Negative Direct Positive Egg-Count	319	97	28			444	36.2
Positive Floatation Negative Direct Negative Egg-Count	74					74	6.0
Positive Floatation Positive Direct Negative Egg-Count	19					19	1.5
Negative Floatation Positive Direct Positive Egg-Count	14	4	3			21	1.7
Negative Floatation Positive Direct Negative Egg-Count	8					8	0.7
TOTAL	606	286	235	53	47	1227	
Total Positive Floatation	584	282	232	53	47	1198	97.6
Total Positive Direct	213	189	207	53	47	709	57.8
Total Positive Egg-Count	505	286	235	53	47	1126	91.8

1227 specimens on which complete information was kept on the results of the three technics employed, 661 or 53.9 per cent were positive on all three, 444 or 36.2 per cent were positive on the floatation and egg-count, negative on the direct; 74 or 6 per cent were positive on the floatation, negative on the direct and egg-count; 19 or 1.5 per cent were positive on the floatation and direct, negative on the egg-count; 21 or 1.7 per cent were negative

on the floatation, positive on the direct and egg-count; 8 or 0.7 per cent were negative on the floatation and egg-count, positive on the direct. Out of 606 specimens of 0–699 eggs per ml., 584 or 96.4 per cent were positive on the floatation, 213 or 35.1 per cent were positive on the direct smear. Out of 286 showing an egg-count of 700–2599 eggs per ml., 282 or 98.6 per cent were positive on floatation, 189 or 66.1 per cent on the direct. Out of 235 in the moderate group of 2600–12,599 eggs per ml., 232 or 98.7 per cent were positive on floatation, 207 or 88.1 per cent on direct. All 53 of heavy infestations of 12,600–25,099 eggs per ml. and 47 very heavy infestations of 25,100 or more eggs per ml. were positive by both technics. No eggs were found on the egg-count in only 101 or 16.7 per cent of the very light infestations. A total of 1198 or 97.6 per cent were positive on the floatation, 709 or 57.8 per cent were positive on the direct and 1126 or 91.8 per cent positive on the egg-count.

Discussion

The early survey of the Rockefeller Sanitary Commission, in 1912, demonstrated hookworm infestations in 83 counties of Texas. They examined 32,151 children of 6–18 years, finding 12,014 or 37.4 per cent positive. Since our survey of 8308 showed 2771 or 33.4 per cent positive, it would appear that there had been only a slight decline in incidence of hookworm in 40 years, however, it must be noted that the early survey included a large number of counties in some of which the incidence was low and our survey included only a small number of counties in all of which the incidence of hookworm is still of importance. A marked decrease of incidence was shown in Jasper, San Jacinto, Hardin, Liberty, Montgomery, Houston, Angelina, and Polk Counties. These counties showed an incidence of 65.2 per cent in 7018 specimens in the early survey but recently showed 32.3 per cent in 5966. This would appear to show slightly over 50 per cent reduction in incidence. However, since the early survey employed only the direct smear method for detection of infestation, the real incidence at that time was much greater. Since we detected only 57.8 per cent of our total positives by direct smear, it would appear that the real incidence in the 1912–14 survey should have approximated 100 per cent. More recent surveys in several southern states, Keller, Leathers and Densen (1940),

have shown that severity increases with incidence. We may assume that the early investigations with high incidence contained greater percentages of heavy infestations and lower percentages of light infestations, a considerable number of which were missed by direct smear. We believe it would be reasonable to assume the real incidence in 1912–14 was between 65 and 100 per cent, more likely 80–90 per cent which would indicate 60–65 per cent reduction in the present survey.

We believe any discussion of the hookworm problem should include San Augustine, Sabine and Walker Counties which were not surveyed but are quite comparable to the surveyed counties in heavy annual rainfall, predominance of pinewoods, sandy soil and sanitary practices of the inhabitants. The 1952 school census for the 14 county area shows approximately 40,000 white children between the ages of 6 and 18. If our findings of incidence and intensity are applicable, probably 13,360 are infested, and probably 3500 would show egg-counts in excess of 2600 eggs per ml. of feces, comprising the moderate, heavy and very heavy categories.

A review of the literature indicates considerable diversity of opinion as to what level of infestation hookworm disease may be expected and should be treated in the interest of the individual and above what degree of intensity should all be treated in the interest of public health. Smillie and Augustine (1926) noted minimal symptoms of hookworm disease in Alabama at 2600 eggs per ml. Andrews (1942) attempting control in Georgia first recommended treatment of individuals with counts of 2000 or more eggs per ml., however, due to observations on the relationship between egg-counts and hemoglobin determinations, he later considered it useless to attempt control where the egg-count was much below 6000 per ml. Hill and Andrews (1942) found a hemoglobin decrease commencing in the 2000–4000 eggs per ml. class and becoming marked at the 4000–8000 eggs per ml. level. At the other extreme, Stiles (1930) thought it the best public health policy to treat all infested individuals.

We believe it would be a conservative estimate to state that there are 1000 children in the 14 county hookworm area of East Texas comprising the heavy and very heavy groups with over 12,600 eggs per ml. No doubt all these would definitely show hookworm disease and should be treated in the interest of their

health. Possibly one-half of the moderate group, 2600–12,599 eggs per ml., another 1000, would show various degrees of hookworm disease. In addition to these, in the interest of public health, at least the remaining 1000 in the moderate group should be treated.

The incidence among negroes was found to compare favorably with that reported in other southern states by Keller *et al.* with the exception of Florida. Both incidence and intensity of infestation indicates no significant problem.

In comparison of the efficiency of the two technics employed for detection of ova, the direct smear demonstrated slightly less than 60 per cent of those found by zinc sulphate floatation. Only 2.4 per cent of our positive specimens were demonstrated by direct smear with negative floatation. Palmer (1941) concurrently employing a special zinc sulphate centrifugal floatation for egg-counting and the Stoll dilution count found an average of over 100 eggs per ml. more by the Stoll method. He attributed this to the failure of degenerate eggs to float in the zinc sulphate. He also showed that there is a significantly greater number of eggs in that portion of a stool first passed than in the rest of the stool. This would indicate less chance of detecting light infestations by direct smear in addition to that of paucity of material capable of being examined by this method. Keller (1934) compared the efficiency of the Stoll egg-count with the direct smear method in the detection of hookworm infestations. He examined over 2400 specimens two times by both methods, finding 7 per cent more positives by the egg-count on the first examination and 4.6 per cent more by that method on the second. He found that 78 per cent of his positives by egg-count were found by direct smear. We found 60 per cent of our egg-count positives on the direct smear. Faust *et al.* (1939), employing an elaborate array of variations of the direct smear and concentration technics, examined 271 fecal specimens. They found 58 helminth infections, less than 10 per cent by direct smear, 86 per cent by their most efficient concentration, zinc sulphate centrifugal floatation followed by loop removal. However, they pointed out that all their infections were light and that only 10 minutes were spent on each preparation.

The foregoing would indicate that the direct smear is of little value in the detection of hookworm infestation, however, in view

of the fact that 91.6 per cent of our positives of 2600 or more eggs per ml., those generally accepted as requiring treatment, were found by this method, it would appear to be of value in surveys aimed chiefly at detecting cases requiring treatment and where the more elaborate concentration technics are not available. This would be the quickest and least expensive type of survey from the standpoint of time and equipment, but it would entail treatment of 35–45 per cent of the light and very light infestations unnecessarily. If time and equipment are available, the Stoll egg-count technic is far superior for the twofold purpose of detection and intensity. If the detection and identification of other intestinal parasites are desired, as is usually the case, we believe the most practical laboratory regime is some type of zinc sulphate concentration followed by the Stoll egg-count on all hookworm positives.

Summary

Incidence of hookworm in eleven east Texas counties was 33.4 per cent of 8308 specimens examined. The range was from a high of 51.3 per cent in Tyler County to 19.2 per cent in Polk County. A negligible incidence of 1.7 per cent was found in 406 colored people. Intensity as revealed by the Stoll egg-count on 2373 positives showed 51.8 per cent very light, 23.5 per cent light, 17.1 per cent moderate, 4.3 per cent heavy and 3.3 per cent very heavy.

A comparison of efficiency of technics employed on 1227 specimens showed 97.6 per cent of positives found by the zinc sulphate floatation method and 57.8 per cent of positives found by direct smear. Although the Stoll egg-count was not employed on specimens negative on the other technics, being used only to determine intensity of infestation, it disclosed eggs in 91.8 per cent of those specimens positive by one or both other technics.

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THE PUBLIC HEALTH IMPORTANCE OF BATS*

J. V. Irons, R. B. Eads, J. E. Grimes, and Alice Conklin

Bats in the United States are generally regarded as beneficial animals as a consequence of their consumption of enormous quantities of insects, many of which are destructive species. However, the isolation of rabies virus from these animals in Florida by Venters *et al.* (1954) and the subsequent reports of Witte (1954), Sullivan *et al.* (1954) and Burns and Farinacci (1954) have necessitated a reappraisal of their significance to man; this is especially true in the southwestern United States where the Mexican free-tailed bat, *Tadarida mexicana*, occurs in enormous seasonal concentrations. The presence of rabies in bats is of concern since they might transmit the infection to foxes, skunks, raccoons and other wild carnivores; to domestic animals such as livestock, dogs and cats; and directly to human beings.

The role played by insectivorous bats in the basic infection chain of rabies in wild life is difficult to evaluate. That the opportunity exists to transmit the disease to other wild animals is evident. We have located and investigated 9 caves in Central Texas in which multimillion colonies of the free-tailed bat maintain summer occupancy. It has been noted that the population levels of raccoons, skunks and foxes is considerably higher in the vicinity of these caves than appears justified on the basis of available food supplies unless bats are consumed. The frequency with which raccoon fecal droppings around the caves contain bat remains indicates that bats are important dietary items for these animals. In the laboratory captive raccoons have been found to feed on bats readily.

Whether or not interspecies spread of rabies is of significance is not known, but this Department has demonstrated natural infection in a wide range of animals (Irons *et al.*, 1954). One hundred and fifty rabid fox were received from 44 counties in 1953; 102 from 34 counties in 1954; and 82 from 36 counties in 1955. Seventy-two positive skunks from 30 counties in 1954 and 60 from 32 counties in 1955 were received. Other positive wild animals seen in the last 10 years have included wolves, bobcats, opossums, raccoons and coyotes.

Rabies is a definite problem in Texas domesticated animals. This department furnishes laboratory confirmation of the diseases in approximately 1,000 dogs and 60 cats annually. Also involved to an appreciable extent in rabies losses are cattle, and to a lesser extent horses, mules, hogs, goats and sheep.

Some indication of the association between bats and pets may be seen in table 1. These examples represent but a fraction of the

TABLE 1

Bat-Pet Associations

Species	Date	Location	Bat Brain Rabies	Remarks
T. mexicana	3-10-56	San Marcos	Neg.	Cat bitten as it played with bat
L. borealis	7-21-56	Austin	Neg.	Cat and dog played with bat
L. borealis	6-19-56	Austin	Neg.	Dog chewing on bat
T. mexicana	8-27-56	Austin	Pos.	Dog played with bat
T. mexicana	8-30-56	Austin	Neg.	Dog bitten by bat
L. borealis	9-20-56	Austin	Neg.	Cat bitten by bat
T. mexicana	9-16-56	Austin	Pos.	Cat climbed tree after bat

total requests which have been received by this Department for rabies studies to be performed on bats rescued from dogs and cats. Due to the press of other duties it has not been feasible to make these tests unless there was also some question of human exposure. While we can cite no cases of rabies in pets definitely attributable to bat bites, it is apparent that bats on or near the ground are fair game for dogs and cats. Since these bats are behaving abnormally, they represent a potential factor in the spread of rabies.

The high level of rabies infection in dogs and wildlife is reflected in the 35 human deaths in Texas in the last 12 years, with 5 of them occurring in 1956. The Texas State Department of Health Biologics Section annually prepares and furnishes physicians of the state in excess of 2,500 14-injection human rabies treatments (Cook *et al.*, 1954). Conservatively estimating this number as one-half of the treatments administered, there are some 5,000 animal exposures a year in Texas of sufficient seriousness to warrant treatment.

As seen in table 2, there have been 22 cases of human bat bites

reported to this Department during 1954–56. Of the 15 bats concerned in these cases, 5 were proven to be rabid and the 8 people involved underwent the antirabic treatment. We know of no untoward effects which were experienced, either from the bites or treatments. Involved in these episodes were 9 free-tailed bats (*Tadarida mexicana*), 5 red bats (*Lasiurus borealis*) and 1 hoary bat (*Lasiurus cinereus*). Two of the *T. mexicana* and 3 of the *L. borealis* were shown to be positive.

TABLE 2
Human Bat Bites

Species	Date	Location	Bat Brain Rabies	Remarks
T. mexicana	5-19-54	Austin	Pos.	Boy playing with bat on school grounds
L. borealis	7-20-54	Austin	Pos.	Girl bitten after bat pulled out of swimming pool
T. mexicana	8-12-54	San Antonio	Neg.	Girl picked up sick bat, bitten
T. mexicana	4-15-55	San Antonio	Neg.	Boy bitten while playing with bat on school ground
L. borealis	6-27-55	Dallas	Neg.	Female bat carrying 3 young fell out of tree, 7 year old boy bitten when adult bat picked up
T. mexicana	2-24-56	Burnet Co.	Not Tested	Adult male bitten on chest while collecting bats
L. cinereus	4-15-56	Lubbock	Neg.	College student bitten while handling caged bat
L. borealis	7- 3-56	Dallas	De-composed	Child bitten on finger
T. mexicana	8- 8-56	Burnet Co.	Neg.	Boy bitten while handling bat at summer camp
T. mexicana	9-14-56	Austin	Pos.	Girl bitten on foot
L. borealis	9-26-56	Denton Co.	Pos.	Teacher and 3 pupils bitten handling bat in class room
T. mexicana	9-26-56	Burnet	Neg.	Lady bitten on finger
T. mexicana	9-26-56	Blanco	Neg.	Adult male bitten while collecting bats
L. borealis	10-10-56	Houston	Pos.	Boy bitten on hand retrieving bat from roof
T. mexicana	10-12-56	Snyder	Neg.	Five children bitten while playing with sick bat

The free-tailed bats are colonial and extremely abundant over much of Texas during the warmer months of the year in caves and in and around buildings. This close colony association with human dwellings represents a potentially dangerous situation. The final answer to eliminating them from buildings is usually

structural modifications to prevent their entry. However, this is not always possible, and under certain conditions colony extermination would appear advisable.

Health authorities are not able to make such recommendations in this state due to the existence of a unique law protecting bats. This law was passed, effective March 10, 1917, making it a misdemeanor (with a fine of not less than $5.00 nor more than $15.00) "to willfully kill or in any manner injure any winged quadruped known as the common bat." This law was enacted on the unwarranted assumption that bats eat appreciable numbers of malaria-carrying mosquitoes. We are recommending that this law be repealed or modified to allow State, County and City health officials to recommend the decimation of premise bats when they become health problems.

L. borealis is a solitary, tree-inhabiting species which is common throughout Texas during the summer months. Although seldom found in numbers, this bat frequently comes to human attention due to the relatively exposed nature of its daytime resting stations in such trees as the pecan and to its striking red coloration. Also, since the females carry their 2 to 4 young until they are able to fend for themselves, the load frequently becomes too much and they fall to the ground to be picked up by pets or children.

The first red bats submitted each year for rabies examination are received in early June,† usually females with young. Females with young have not been received after the first week or two in July. Only an occasional red bat is submitted after July, although one was sent in from Wilson County in South Texas as late as October 31, 1956. In 1954, 13 red bats were tested for rabies; 42 in 1955 (31 from Dallas and Dallas County) and 40 in 1956 (34 from Austin and Travis County). Rabies virus was isolated from 5 of these 95 red bats.

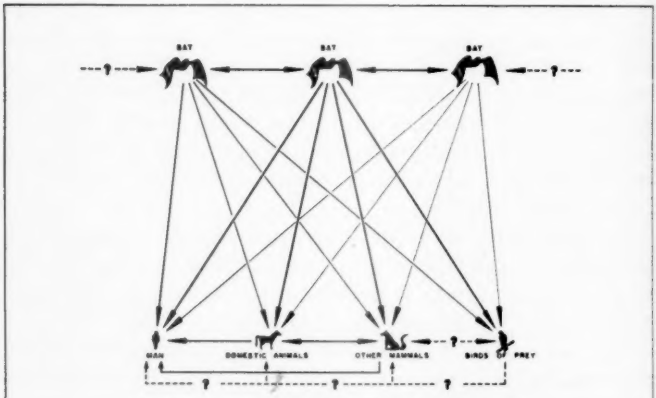
Although other states have reported that bats subsequently shown to be rabid made unprovoked attacks on human beings all of the cases reported here have resulted from the bats being handled.

That these animals are of public health significance has been demonstrated by 2 deaths in Texas believed to have resulted from exposure to bats. The first was reported by Sulkin and Greve (1954) and concerns a woman from Big Spring who died in

October of 1951. Sixteen days prior to onset of illness she examined what she thought to be a dead bat and was bitten on the forearm. The demonstration of Negri bodies in central motor neurons strongly suggested that death resulted from rabies. Bat bite was the probable source of infection as there was no history of other animal exposure.

The second rabies death occurred January 4, 1956, with the death of George C. Menzies of the Entomology Section, Texas State Department of Health, a member of our team conducting bat rabies investigations. Onset of clinical symptoms was sudden. Mr. Menzies spent January 1 in a city some 100 miles from Austin. He appeared well and no one suspected he was ill until returning home that night he requested that his wife drive as he was experiencing vision impairment. The night was spent in fitful sleep interrupted by nightmares. On January 2 he awoke early and upon getting up and trying to drink water underwent 2 convulsive seizures. A physician was called and the patient volunteered the information that he had rabies.

Outstanding symptoms were throat muscle paralysis, respiratory difficulty, acute anxiety and apprehension. As breathing became more labored and swallowing difficult and painful, the patient was hospitalized the afternoon of January 2. Supportive therapy was of no avail and Mr. Menzies expired the afternoon of January 4, 1956.



BAT RABIES — EPIDEMIOLOGICAL CHAIN OF INFECTION

J. S. Wiseman

No viral agent was found in saliva inoculated in mice and no Negri bodies were demonstrable in stained impression smears of G. C. M. brain tissue. Six adult mice and 6 infant mice were inoculated intracranially on January 5 with 1:1,000 dilutions of brain material. On January 11, symptoms of rabies were noticed in adult mice. One was sacrificed and Negri bodies found in impression smears; on January 12, 3 of the adult mice died and Negri bodies were demonstrated. The remaining 2 adult mice died of rabies the following day. The infant mice were moribund on January 16 and impression smears of brain tissue contained Negri bodies. Virus serum neutralization tests confirmed the fact that the isolates in both adult and infant mice were rabies virus. No viral agent was recovered by the inoculation of mice with G. C. M. spleen, liver and lung tissue.

Mr. Menzies was not able to throw any light on the method whereby he contracted rabies, giving no history of bat or other animal bites. He was primarily concerned with field investigations into the ecology of the Mexican free-tailed bats in the Central Texas caves. Several months prior to his death he had bled approximately 75 free-tailed bats for serological studies and participated in the experimental inoculation of 25 free-tailed bats with rabies virus. He also maintained a colony of these bats in the laboratory.

In the field Mr. Menzies handled a tremendous number of bats while wearing rubber gloves. In April, May and June, 1955, he and several associates banded some 10,000 in connection with migration studies. On November 15, 1955, he made a trip to Frio Cave in Uvalde County during the course of which several hundred bats were examined in a search for banded specimens. On October 20, 1955, he made a trip to Bracken Cave in Comal County to look for banded bats and Wilson Cave in Kerr County was visited October 13, 1955, for the same purpose.

In retrospect, it has been postulated that Mr. Menzies acquired the infection through the contamination of his gloves and the accidental rubbing of his neck at the site of a chronic skin eruption.

Summary

In summary, 22 human bites have been reported to this Department during the period 1954–56, plus numerous dog and

cat-bat associations. There have been 2 human deaths attributable to bat exposure in Texas, one of which is discussed in some detail. Bats definitely represent a public health problem but the extent of their involvement in the basic infection chain of rabies in nature remains to be clarified.

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THE FATE OF THE CHROMIUM-51 LABEL OF AUTOLOGOUS HEMOGLOBIN AND PLASMA IN RABBITS*

WILLIAM C. LEVIN, JOHN E. PERRY, T. G. BLOCKER, JR.

The use of chromium-51 in the chemical form of sodium chromate has become a popular method for the study of erythrocyte mass and erythrocyte survival (1–8). The procedure commonly used involves the injection of washed tagged erythrocytes or tagged whole blood in which the excess chromate has been reduced to the trivalent form by the addition of ascorbic acid. The hexavalent chromium ion enters the erythrocyte and tags the globin molecule; trivalent chromium has been demonstrated to tag the proteins of the plasma (2). This study deals with the fate of the hexavalent chromium label of hemoglobin resulting from the destruction of tagged erythrocytes and the fate of the trivalent chromium label of the plasma proteins.

Fate of the Hexavalent Chromium Label of Hemoglobin

Massive doses of Cr-51 tagged hemoglobin were injected into a human subject by Ebaugh *et al.* (1) in an experiment to determine whether or not bound Cr-51 would tag erythrocytes *in vivo*. The results indicated that no tagging of erythrocytes occurred and that most of the radioactivity appeared in the urine. Recently, Jandl *et al.* (3) demonstrated that the Cr-51 label is concentrated to a large extent in the liver after a single injection of labeled hemoglobin. Since a massive dose of hemoglobin simulates neither the normal state nor commonly encountered hemolytic states, it was assumed that multiple small doses injected over a period of six days would provide a more valid representation of the fate of the hemoglobin label.

In the experiment ten male and female albino rabbits were used. Ten milliliters of heparinized blood were removed from the heart of each rabbit and tagged with approximately fifty microcuries of Cr-51 in the form of sodium chromate. The chromium which still remained in the plasma after 30 minutes was reduced with ascorbic acid. The cells were washed three times

in physiological saline, re-suspended in saline, and hemolyzed by alternate freezing and thawing. One milliliter of hemoglobin solution was equivalent to approximately 0.5 ml. of packed erythrocytes. One milliliter of autologous hemoglobin solution was injected intravenously via the marginal ear vein daily for six consecutive days. The rabbits were kept in a metabolism cage, and urine and feces were collected quantitatively. Blood was drawn at various intervals to determine the radioactivity of the erythrocytes. The rabbits were sacrificed 8 to 32 days following the first injection, and the radioactivity in the various organs was determined with a Texaco High Efficiency Gamma Ray Detector.

The results were expressed as the percentage of the total injected dose of radioactivity present in each organ. On the 8th day following the first injection the liver contained 5 per cent of the activity; thereafter, the liver content increased rapidly to 11 per cent on the 12th day. A plateau was attained and 9 per cent of the injected activity was still present in the liver on the 32nd day. In the kidneys 7 per cent was detected on the 8th day. This decreased slowly to 2 per cent on the 16th day; 1.5 per cent of the activity was found in the kidneys on the 32nd day. The activity in the small intestine remained constant at a level of 0.8 per cent and in the large intestine the level was constant at a level of 1 per cent. The activity in all other organs never exceeded 0.2 per cent of the total injected dose. No significant amount of radioactivity was demonstrated in the erythrocytes at any time.

The radioactivity in the urine increased rapidly until 6 to 10 days after injection of the labeled hemoglobin and then increased more slowly to reach a plateau. On the 6th day the average cumulative amount excreted was 32.3 per cent of the injected dose with a range of 22 per cent to 46 per cent. The cumulative amount excreted rose slightly and attained a value between 34 to 59 per cent at 32 days. The average amount excreted in the feces was 4.1 per cent with a range of 3 per cent to 6 per cent.

Fate of the Trivalent Chromium Label of Plasma Proteins

In 15 albino rabbits 20 ml. of heparinized blood were drawn from the heart and centrifuged under sterile conditions to separate the plasma. Approximately 50 microcuries of Cr-51 as sodium chromate were added to the plasma followed by 25 mg. of non-buffered ascorbic acid. After 20 minutes the plasma was re-

injected into the same rabbit intravenously. The rabbits were placed in a metabolism cage and the urine and feces quantitatively collected. Blood was drawn at different intervals to determine the radioactivity of the erythrocytes. The rabbits were sacrificed at various intervals, the organs were perfused with normal saline solution, and the radioactivity determined as before.

A variable relationship between levels of radioactivity and time intervals following injection was noted, but in general the liver contained 0.3 per cent of the injected dose of radioactivity on the first day after the injection and this slowly increased to 2.1 per cent on the 15th day. The large and small intestines each contained 0.02 per cent on the first day and 0.14 per cent on the 15th day. The amount of radioactivity present in the other organs was too small to measure.

The amount of radioactivity excreted in the urine one day after injection averaged 52 per cent of the injected dose with a range of 28 per cent to 70 per cent. The urinary activity gradually increased an average of 10 per cent to 15 per cent and a cumulative average of 63 per cent was attained on the 15th day. The feces the first day contained an average of 2.2 per cent of the injected dose with a range of 1 per cent to 8 per cent. This level of fecal excretion was maintained for 15 days. None of the radioactivity was detected in the erythrocytes. These data agree roughly with those obtained by Visek *et al.* (9) after injection of radioactive $CrCl_3$ in rats.

Summary and Conclusions

1. Following hemolysis of erythrocytes tagged with Cr-51 as sodium chromate, the end-products of hemoglobin catabolism which carry the Cr-51 label appear to be concentrated in the liver and are rapidly excreted in the urine.

2. The radioactive Cr-51 label of plasma proteins is rapidly excreted in the urine and to a lesser extent in the feces without concentration in the liver or kidneys.

3. No re-utilization of Cr-51 for erythrocyte labeling was demonstrated following the use of this isotope in the tagging of either erythrocytes or plasma proteins.

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AEQUANIMITAS OR ATARAXIA

WILLIAM P. LONGMIRE, JR.*

In May, 1889, Sir William Osler delivered the Valedictory Address at the University of Pennsylvania which he entitled "Aequanimitas." Aequanimitas was to Osler a mental attitude of calm equanimity which enables one to rise superior to the trials of life. Some six years later on the occasion of his departure from America to assume the Chair of Medicine at Oxford University, Osler voiced, "I have had three personal ideals. One, to do the day's work well and not to bother about tomorrow. Second to act the golden rule, and the third has been to cultivate such a measure of equanimity as would enable me to bear success with humility, the affection of my friends without pride, and to be ready when the day of sorrow and grief came to meet it with the courage befitting a man." Such was the philosophy of life which this wise physician expressed over and over in his written and spoken words. It was the development and cultivation of this inner calmness which was one of the great principles of living that the father of modern American medicine aspired to impart to his students and associates. It was a way of life, an attitude, that could not be assumed over night, but was an inherent part of a man's behavior that had to be nurtured slowly to maturity by active conscious effort and discipline.

Osler's era was a period of momentous change in American medicine. The Flexner Report had stimulated a complete reorganization and revision of the medical schools in America. The majority of the members of the medical profession, both young and old, realized the need for improvement and were eager to follow the ideas of the leading physicians of their day. They wanted someone to tell them how to improve and to show them the way. Few men could have had the talents for this role that Osler possessed. He was well acquainted with medical progress throughout the world, and he wished to graft the best institutions of that day onto the American Scene. As he said, "It has been my ambition to build up a great clinic on Teutonic lines, not on those

* Professor of Surgery, University of California, School of Medicine, Los Angeles. Received December 14, 1956.

previously followed in this country and England, but on lines which have proved so successful on the continent, and which have placed the scientific medicine of Germany in the forefront of the world." Much of his advice, which was so widely heralded, seems also to have been influenced by continental and more specifically Teutonic behavior. "The master word is work," "success during the first ten years means endurance and perseverance," and "happy lives shall be yours, because busy and useful; having been initiated into the great secret—that happiness lies in the absorption in some vocation which satisfies the soul; that we are here to *add* what we can to, not to *get* what we can from life." "A young man married is a young man marred," "In plain language, put your affections in cold storage for a few years, and you will take them out ripened, perhaps a little mellow, but certainly less subject to those frequent changes which preplex so many young men."

One can not help but wonder what the reactions of this wise man of medicine would be to our present standards should he return to our medical institutions and particularly our medical schools today: Institutions geared to a 40 hour week, and with some unions seriously considering a 4 day week; many medical students and most house officers married and with families; young surgeons with 5 years of postgraduate training and study standing for certification as specialists in their chosen field with the barest knowledge of who Billroth, Paré, or even Osler's associate Halsted were, or what their contributions to surgery may have been. What of the almost universal concern in the mind of the young physician to "put in" the number of years' training required by the board and get into practice.

In 1894, Osler writing in a publication of the University of Pennsylvania refers to the delightful ataraxia of the great naturalist, Joseph Leidy. Would he be surprised today to find this term being widely distributed in the drug house literature of the "detail man"? A whole family of ataraxic drugs attempting to tranquilize our modern society; a person trying to gain in 5 minutes from a Miltown tablet what Osler wished to instill in a man's character through years of discipline and effort. In our time, one might even be led to believe that ataraxia had replaced aequanimitas. Undoubtedly, such observations make most of our senior citizens, even of today, pause to reflect, and one might anticipate

that a wise man from a half century past would need more than a moment to gather his wits.

I am not one to believe, however, that the ghost of Osler would give one anguished gasp at our modern demoralized civilization and retreat to his moldy crypt, thankful that the scythe of time had spared him this era of ruin, for he also taught that life is change and the changes would stimulate spirit and enthusiasm in Osler. But, just as the body still needs food to eat, water to drink, and air to breathe as it did a half century ago, he would still insist that there are certain ideals and fundamental values which are imperishable truths.

Leaving aside the enormous scientific advances which have occurred in medicine in the past half century, many of the changes in our way of life as men of medicine in this country have been byproducts of the wealth, the development, and the prosperity of our great nation. Halsted is generally considered the father and originator of the surgical residency system so currently prevalent throughout this country. Although I can quote no authority for the statement, it seems quite probable that Halsted's original intent was merely to introduce the "oberartz" system of the Viennese and Germanic schools into America. In fact, his early senior residents did stay on for several years. The opportunities for his well-trained residents elsewhere in the country were soon so enticing, however, that none of them found it necessary to complete the long 10 to 20 year oberartz period customary in the Teutonic universities after which the system was patterned. Soon, the system of a year of senior residency was established, and this has been the pattern generally adopted throughout the country. Using the surgical group as an example, in no other country in the world today are such opportunities available as are presented to the young surgeon completing his training in America. Each year, roughly 500 new doctors complete their surgical training and qualify as specialists in general surgery. Some say that this is not enough, others cry loudly of oversaturation, but governed largely by the law of supply and demand, the rewards seem sufficient to attract rather consistently this number of young doctors into the field. Once so certified and placed on a hospital staff, they soon have the same rights and privileges in regard to the care of their patients that the most senior staff surgeon enjoys. Although, with the exception of a

few fortunate individuals, most of these men can look forward to a few lean years, they have a free and equal opportunity to attract their share of the medical or surgical practice of the community. How different this is from the lot of the young surgeon in other countries of the world.

In 1950, I had an occasion to spend two months visiting medical schools in one of the European countries with a group of faculty members from various American medical schools. As part of our duties, we were asked to review their system of medical education and training in the particular speciality in which we were interested with a view toward possibly introducing somewhat more democratic methods into their program. As is so often the case when viewing a complicated and intricate problem, at the end of the first week there seemed to be many helpful suggestions; at the end of two months, however, when we had become fully acquainted with all of the ramifications of the problem, our suggestions and recommendations were, to say the least, meager in number and somewhat less than significant in import. For example, my first reaction was that the American system of a graded surgical residency program should be introduced. It soon became apparent, however, when we looked beyond the level of the medical schools, that there would be no place for these well-trained young surgeons to go. Every position in the country in which a man could actually expect to practice surgery was filled, and opportunities for new surgeons appeared only upon the death or resignation of the incumbent surgeon.

Further experience abroad opened our naive eyes to the fact that such was the situation in all countries throughout the western world, and that America alone, with its constantly expanding economy, could absorb the tremendous output of trained surgeons produced by a widely adopted residency training program.

Such, of course, is only one small facet of the total medical picture, but it portrays so clearly the unique opportunities afforded the young man in American Medicine today.

In 1954 while attending the Western German Surgical Congress in Munich I had an occasion to have a rather lengthy discussion with a Communist who was the Chief Surgeon in a large hospital in the Russian zone of Germany. His wife had shown her loyalty to the party by spending two years with pick and shovel

helping to dig the Moscow subway. In his position as Chief Surgeon of a 400 bed surgical clinic, he received $119.00 per month. He was quite proud of the new 400,000 mark wing which had just been added to his hospital. He said they were told behind the iron curtain that thousands of additional hospital beds were needed in America but that we spent so much on luxuries that this country could not afford such vital things as new hospitals.

He told me that under their soviet supervised system there were three paths that might be followed to become a medical doctor:

1. Normal progression, year by year, through the regular school system. If a student's grades were sufficiently high, or if he had engaged in some outstanding political activity, he might be selected to enter medical school. Sixty per cent of the members of each class must come from the families of farmers or laborers.

2. A child might receive his primary education, then enter a factory or start to work on a farm. After a period of a few years, the child might be selected to return to a workers' College where an attempt is made to summarize in a short time the years of schooling he had missed, and it was possible for some of these students to go on into medicine. One or more children in a factory, depending upon the size of the factory, might be selected for this additional education.

3. A nurse may be permitted to take some additional medical education and receive a limited type of medical degree which permits her to practice medicine. Or the nurse may complete the regular medical school course and become a full pledged medical doctor.

He said they had been very disappointed in some of the children of peasants and laborers who had been educated. For, as has been demonstrated for years in America, when these children were educated they frequently developed capitalistic ideas.

There was a tone of disillusionment in his voice as he spoke further of the young people of the East. "The young people all look to the West," as he said, "it all starts with 'Boogie Woogie' and prevails all of their thinking. To them there seems to be fun, joy, and opportunity in the west regardless of how well they have been reared politically in the east." "But" he continued, "who could blame them? We have little of materialistic value to offer them in Communism at present. All that we can offer them

is hard work, idealism, sacrifice, and hope for the future. We are attempting to create a utopia here on earth." He repeatedly spoke of better times in the future, but failed to explain what event, or events, would improve their present situation in the future.

I asked him if the Soviet attempted to influence their affairs and his answers reaffirmed the complete domination of Russia over the entire life of the satellite people. All of the people were forced to attend weekly lectures, the subjects of which constantly propagandized the superiority of the Russian people and constantly reminded them that all wisdom and culture are centered in Moscow. My friend said he was at times ordered to give such lectures and he was instructed to give his audience misinformation about the discovery of penicillin and insulin in Russia and many other Russian credits of doubtful authenticity. Some of the lectures were so ridiculous he refused to give them for, as he said, these were just the methods which the Nazis used.

So much for a quick glimpse behind the iron curtain. We may hope there are more substantial attractions on this side of the curtain than fun and boogie woogie.

It has been said many times that with authority goes responsibility, and the same might be said of our opportunities. With opportunity goes obligation; an obligation not to fritter away one of the greatest natural resources of our nation—the energies and abilities of our young people in medicine. Are we to drain these people through medical school, a few years of postgraduate training, and into a utilitarian practice without any thought of stopping along the way for a time, or permanently perchance, for them to contribute to the replenishing and restocking of the fountain of knowledge from which they have had their drink.

Not long ago a fourth year student stopped in my office to ask for a letter of recommendation to support his application for internship in another service. He said he really wanted to go into surgery, but after thinking it over he thought that surgery would be so demanding of his time that he would not have enough time to be at home with his family. Now I don't think that every student should consider being a surgeon, and I'm sure that the wives of most medical students would warm to such a statement, but I could not help thinking to myself "Has this young man or his wife ever read Osler's "A Way of Life."

Times have changed, conditions have changed, the social order

has changed, but let us not forget the unchangeable truths and ideals which must live on generation after generation. Fortune has looked on all of us with a broad grin and has offered each of us unparalleled opportunities. The preservation and activation of these fundamental ideals is our obligation: Dedication to a worthwhile vocation, work, self-discipline, and a desire to contribute *to* and not only take *from* life. In so doing, let us not substitute a modern tranquilized ataraxia for good, old-fashioned aequanimitas.

SPECIAL USES OF THE MULTIPURPOSE TISSUE CULTURE CHAMBER

GEORGE G. ROSE*

A multipurpose culture chamber was introduced recently (Rose, 1954) as an aid to tissue culture studies in which phase contrast and interference contrast analyses were to be made. Perfusion procedures and simple fluid exchangings were carried out with needles which pierced the rubber gasket (Fig. Ib). The chamber was completely separable so that the coverslip cultures could be fixed, stained and mounted. Two reports (Rose, 1955, 1956) have been made based on studies which utilized this chamber as the major culturing vessel. Other projects currently in progress have made further use of this sandwich-type chamber plus variations in the basic design which are reported herein.

Compression Chamber. It was desirable to introduce a positive pressure upon cells cultivated *in vitro* in a manner which would permit them to be viewed and photographed through phase-contrast optics. This situation introduced a problem relative to the thickness of the glass. With bright-field optics this was not so important but with phase-contrast methods coverslip thinness was a critical requirement. Thick re-inforcing glass or plastic plates over the number one thin coverslip walls of the chamber sharply reduced the quality of the phase system so that it was only partially effective with the 10x objective and completely ineffective with the higher magnification lenses. Strips of two-inch-wide cellophane tape were cut to the width of the coverslips (43 x 50 mm.). These were placed over the two opposing coverslips (Fig. Ia) as the chamber was fabricated. After the chamber was completely assembled positive pressure of a desired and measurable amount was introduced into the chamber with a syringe. This technique allowed the coverslip walls to remain thin enough for the use of phase objectives. The phase display was unaltered through the 10x objective and its quality was only slightly reduced with the 21x, 43x and 97x objectives (B & L, long-working-distance condenser). Similarly negative pressure studies are being investigated in *tension chambers*.

Colloidal Screen Chambers. This variation was used in constructing dialyzing and ultrafiltration culture chambers. In assembling the multipurpose culture chamber two rubber gaskets were used in place of the usual one. A sheet of cellophane the size of the coverslips and of known porosity was interposed between

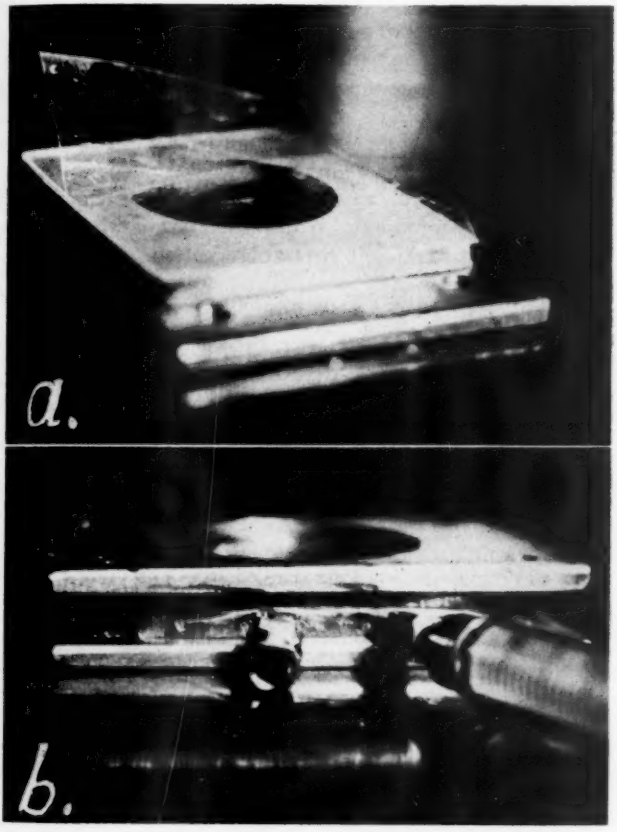

FIG. I. (a) Assembly of a compression chamber. Note the piece of cellophane tape partially applied to the top coverslip. (b) The colloidal chamber with vent and syringe needles inserted into the gasket below the cellulose membrane.

these two gaskets. This procedure formed two adjacent chambers with a common cellophane wall (Fig. Ib). The culture to be observed was established with the construction of this variation or a culture from the conventional single-vaulted chamber which had been initiated earlier was converted to this type. The now thicker chamber was still amenable to phase contrast observation. Sera, other biological fluids or cultures could be introduced into the vault opposite the culture of study. The culture was then observed while influenced by the dialysate.

If the coverslip of the vault opposite the observed culture was covered with cellophane tape as in the *compression chamber* an increased pressure could be introduced into this side. The culture-side vault required a compensating vent needle during the pressurizing period. After the additional fluid was added to the pressure side the vent needle of the culture side was removed. This arrangement then produced an ultrafiltration culture chamber which also permitted phase-contrast observation.

NOTES AND PAPERS CITED

* From the Tissue Culture Laboratory, Hermann Hospital and The University of Texas M. D. Anderson Hospital and Tumor Clinic, Houston, Texas, Tissue Culture Laboratory, The University of Texas Medical Branch, Galveston, Texas. Aided by a grant from the American Cancer Society. Accepted for publication January 16, 1957.

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OBSERVATIONS ON THE DYNAMICS OF PINOCYTIC AND *VARIANT PINOCYTIC (VP) CELLS* IN GEY'S HUMAN MALIGNANT EPIDERMOID STRAIN *HELA*

GEORGE G. ROSE*

Potocytosis, cell sipping or submicroscopic drinking was suggested at the dawn of the century (Meltzer, 1904). It was another quarter of a century before the motion picture studies of macrophages in tissue culture was revealed by Warren H. Lewis. In his first publication which so graphically introduced the physiological action of the cell (*pinocytosis* or cell drinking), Lewis (1931) pointed out the comparable features of the sister function of the cell (*phagocytosis* or particulate cellular ingestion).

Recently Palade (1953), through electron-microscopic studies on the endothelial cells of blood capillaries found spherical vesicles (650A) concentrated under the cell membrane. Many of these vesicles appeared to open at the surface of the cell membrane and may have represented what Meltzer earlier had envisaged as *potocytosis*.

Lewis did not see an accumulation of granules in these pinocytic cells and he was fairly certain that pinocytosis was not a degenerative phenomenon. He concluded that *pinocytosis* was not necessary for survival but simply represented with *phagocytosis* a special intracellular digestive ability.

Rotating nuclei were also observed by Lewis (1) (1937) in pinocytic malignant cells but were not directly linked as a collaborating mechanism. Pomerat (1953) re-emphasized nuclear rotations. His observations were frequently made in cells which were demonstrating *pinocytosis*.

Though Lewis (1937) saw many sarcoma cells undergoing *pinocytosis*, the incidence in normal fibroblasts was very low. From other reports (Lewis (2), 1937; Lewis *et al.*, 1949; Pomerat *et al.*, 1954; Gey *et al.*, (6); Gey *et al.*, (7); Gey *et al.*, (8), Rose *et al.*, 1951 and Pomerat, 1953) and from those already cited it would appear that *pinocytosis* was commonly though not exclusively seen in tissue cultures of (1) cells of benign and ma-

lignant epithelial origin, (2) macrophages from normal and malignant tissues and (3) sarcoma cells.

Gey (Gey *et al.*, (6, 7, 8)) concisely re-described pinocytosis as viewed under phase-contrast optics. More recently, a variation of the pinocytic process was described (Rose, 1955; Rose, 1956) in epithelial cells of Gey's strain *HeLa* (Gey *et al.*, 1952; Scherer *et al.*, 1953). This stable strain of cells which was derived from a human epidermoid carcinoma of the cervix followed the pinocytic patterns of activity illustrated first by Lewis as long as they were in a nutrient basically composed of ascitic or pleural fluids. When the nutrient was shifted to one basically composed of human sera the variation occurred in some or all of the pinocytic cells. This alteration was simply described as a *variant pinocyte* or *VP cell* whose production was stimulated by the nutrient shift from ascitic or pleural fluids to sera. These *VP cells*, unlike the pinocytes noted by Lewis, did accumulate granules in the cytoplasm. These granules were of unusual size and ranged from 4–25 *micra* in contrast to juxtanuclear granules of other pinocytes which usually measured 1–3 *micra*. The mechanism involved in the production of the *VP cell* was not well understood in the original report and only some additional light has been shed with the help of the time-lapse movie.

Materials and Methods

Culturing. Stock cultures of Gey's strain *HeLa* were maintained in roller tubes at incubator temperatures (37.0° ± 1.5° C.) in a nutrient composed of benign or malignant ascitic or pleural fluids and Earle's balanced salt solution in equal amounts. These nutrients also contained chicken embryonic extract 3–4 per cent (EE50, Microbiological Associates), Penicillin G 1,000 units/cc. (Squibb) and phenol red 0.001 per cent. Occasionally nutrients were formulated with 100 per cent ascitic and pleural fluids. Circular colonies of cells were readily cultivated directly upon the glass walls of the roller tubes using this nutrient formula. Sheets of cells were removed with a narrow stainless

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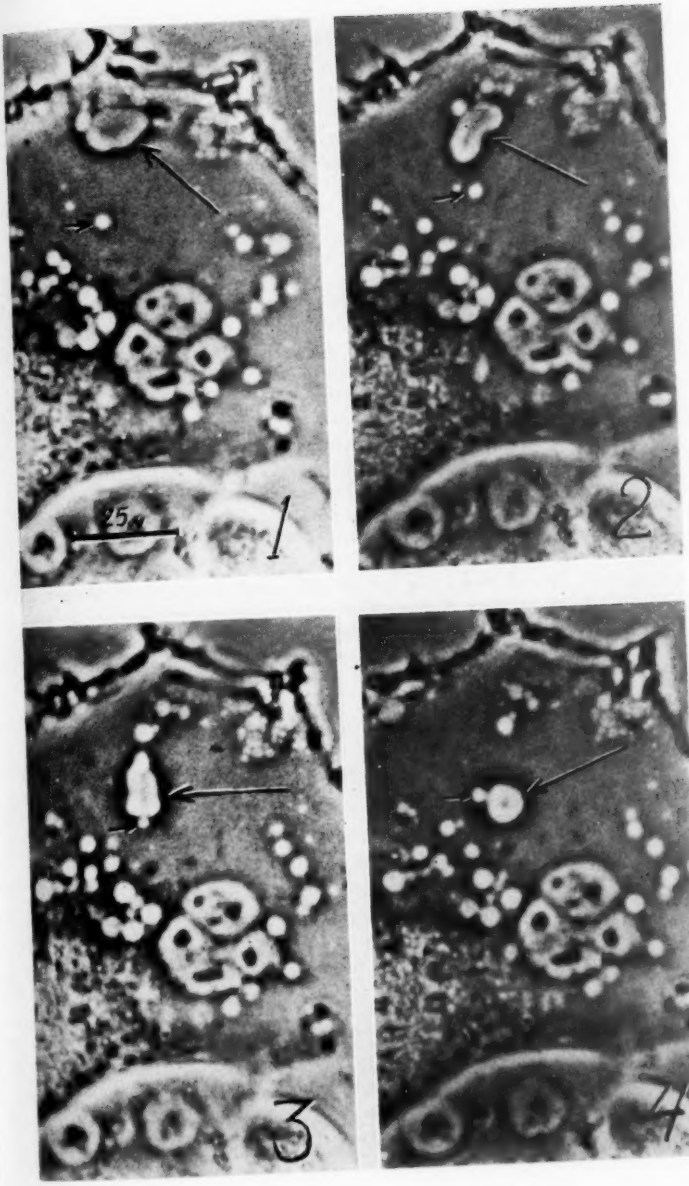
FIGS. 1 through 4. Pinocyte of strain *HeLa* cultivated in a basic nutrient. Series taken from the 16 mm. time-lapse-cine records *(Phase contrast)*. The long arrow points to one droplet which suddenly was released from its position inside of the cell membrane to the juxtanuclear area. Note that the droplet ahead of this one (short arrow) was pushed aside, the large droplet rapidly shrunk then rounded and that its sudden thrust toward the nucleus ceased. The sequence represents about 90 seconds of life.

FIG. 2. The "peanut" form in this photograph seemed to indicate that the droplet was truly penetrating an invisible barrier.


25μ
1
2
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steel spatula and poured into Petri dishes, the 1–2 mm. sized resultant fragments were sub-cultured into the multipurpose culture chambers (Rose, 1954). Single colonies of cells were cultivated in each chamber with the same nutrient used in the roller tubes. After the chamber cultures had attained a diametric size of 5–6 mm. the initial nutrient was replaced with a human serum nutrient. These serum nutrients did not contain the embryonic extracts and were formulated in 25 per cent, 50 per cent and 100 per cent serum portions, the balance composed of Earle's salt solution.

Observation. Multipurpose culture chambers were viewed with phase-contrast optics (Bausch & Lomb) which employed a long-working-distance condenser. Along with frequent visual observations the time-lapse 16 mm. movie camera (EMDECO) and time-lapse still photographic camera were used to uncover much of the information related in this report.

Staining. Several staining methods and histochemical tests were used but two approaches particularly were found satisfactory and they both involved neutral red. For vital staining the chambers were evacuated and filled with a freshly prepared solution of neutral red (0.05 per cent) in Earle's balanced salt solution. After two minutes the neutral red was removed and the chamber refilled with the nutrient. To prepare a permanent mount the chamber was refilled with Earle's formol after the neutral red solution had been withdrawn. Fixation was carried out for thirty minutes, after which time the cultures were washed in distilled water and then counterstained in Delafield's hematoxylin. After adequate water washes the cultures were dehydrated in acetone, cleared in xylene and mounted in a plastic mounting media. The staining procedure could be carried out entirely in the chamber but because of the affinity between the dyes and the rubber gaskets it became more practical to carry out these procedures after the coverslips had been removed from the chambers.

Results

Pinocytosis. In order to understand fully the *VP cell* formation it was felt that a basic knowledge of pinocytosis in the strain *HeLa* under the influence of the initial nutrients was first mandatory. *Pinocytosis* in circular colonies was predominantly a peripheral activity where the cells displayed free membrane borders. Colonies were scanned with the 10x objective and the cells undergoing major *pinocytosis* (Figs. 1–8) were counted. *Pinocytosis* was considered "major" when easily visualized with such

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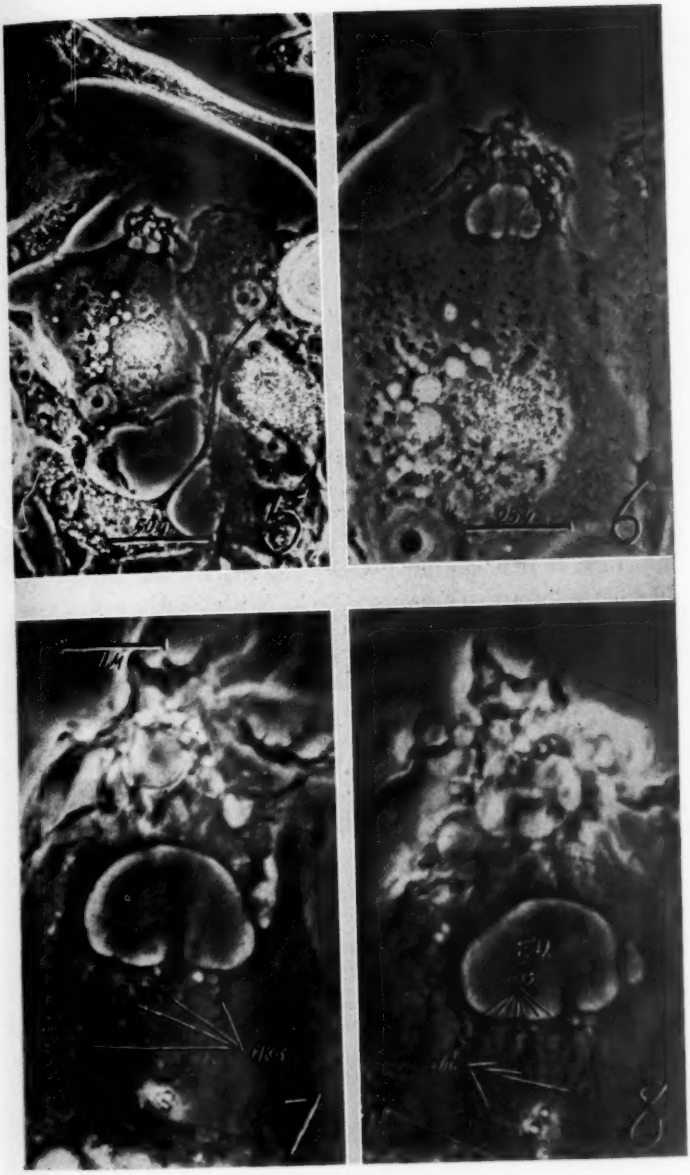
FIGS. 5 through 8. Pinocyte of strain *HeLa* cultivated in a basic nutrient. Photographs were taken at seven minute intervals *(Phase contrast)*.

FIGS. 7 and 8. Note the affinity of the *microkinetospheres* (MKS) for the fluid dropet (F.D.) as opposed to the general distribution of the mitochondria (Mit.).



a low-powered lens. These cells demonstrated fluid droplets in the range of 5–10 *micra*. This was a very effective way of determining the entire pinocytic activity of the culture. Many additional pinocytic cells were seen with the 21x objective but their numbers were closely proportionate to those seen with the 10x. A culture which demonstrated 5–10 "major" pinocytes with the 10x objective one day, often contained only 1–5 the next day. When this shift toward a decrease of pinocytes was noted, there was usually a concomitant increase in epithelialization which was frequently arranged in a very regular pattern. When the basic nutrient was exchanged there usually followed an increase in the number of total pinocytes. In a few days the pinocytes again waned and were followed by the advancing epithelial emigration. Too, it was observed that if a culture had it's nutrient exchanged with a fluid from a chamber whose *pinocytosis* activity had subsided the pinocytic wave was measurably less than if a fresh nutrient were added but, nevertheless, showed good epithelialization.

Individual pinocytes viewed with the eye through 21x phase objectives at first appeared static. The motion was barely perceptible at the active membrane areas. With the 43x and 97x objectives some accentuation in the activity was demonstrated. The limiting membrane even with the 97x objective appeared extremely sluggish as though it were moving against an almost equalizing force. Fluid droplets entered these cells from one or several points around the cell. *Pinocytosis* was not observed as a feature of the entire cell's perimeter although occasionally a cell would have four or five areas receiving fluid droplets.

Time-lapse studies were made using projection sequence ratios of 60x and 120x the normal speed of life. In viewing these films the sluggish pinocytes were so accentuated that their motions

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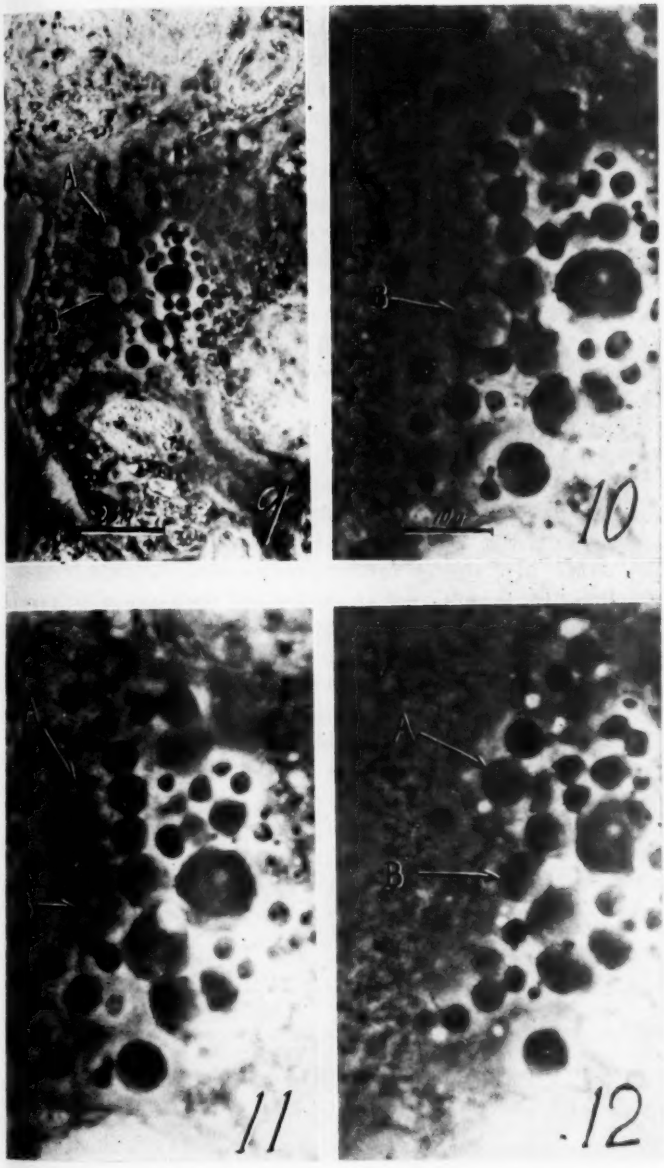
FIGS. 9 through 11. Peripheral edge of a *VP cell* of strain *HeLa* cultivated in a serum nutrient and photographed at five-minute intervals *(Phase contrast)*. These figures define the transition of the incoming fluid droplets into the *VP granules*.

FIG. 9. Low power view which displays two incoming fluid droplets (*A* and *B*).

FIG. 10. Droplet *A* has become gray through the phase optics and, therefore, appears to blend with the cytoplasm. Droplet *B* is slower to shift its shade and still appears white.

FIG. 11. Droplet *A* returns to view as it darkens.

FIG. 12. Twenty minutes after figure 11 was photographed both droplets *A* and *B* were viewed as black granules *(VP granules)*.


A
B
9
10
11
12

seemed to jump to life in an unrealistic though a now perceptible and analyzable manner. The limiting membrane at the active zones rhythmically produced a fanning and cupping motion which in some manner engulfed droplets from the fluid medium. These droplets which appeared white (B & L phase) generally measured less than one micron but rapidly coalesced with neighboring droplets to form spheres ranging up to ten micra. After this initial coalescence which occurred just inside the limiting membrane the now larger droplet was released rapidly into the cytoplasm and hurriedly crossed toward the juxtanuclear zone. Occasionally a large droplet which measured several micra apparently was admitted to the cell (Figs. 5–8) without a coalition phenomenon. Many of these enlarged droplets seemed to penetrate an invisible barrier some 5–15 *micra* within the peripheral edge of the cell and then burst into the cytoplasm (Figs. 1–4). These droplets were immediately contacted by minute granules *(microkinetospheres)* which rushed out from the juxtanuclear zone (Figs. 7–8). As the droplets crossed the cytoplasm and moved centrally they usually decreased in size quite rapidly (5–20 minutes) though often re-coalesced during this transit. Finally they shrank, and if no further coalescence occurred, shifted their phase visual display to black as they became lost as minute granules in the juxtanuclear aggregate. An occasional droplet failed to shrink until after it had lingered for some time near the nucleus or juxtanuclear aggregation. Shrinkage of the lingering droplets was often delayed an hour or more. Filamentous mitochondrial activity was present throughout this process though only speculative action could be assessed to this interplay. Occasionally, mitochondria appeared to be arranged in parallel tracks along which the droplets migrated. These apparent mitochondrial guides were also used by the outrushing *microkinetospheres* which contacted the incoming droplets.

Visual observations through the 43x and 97x objectives demon-

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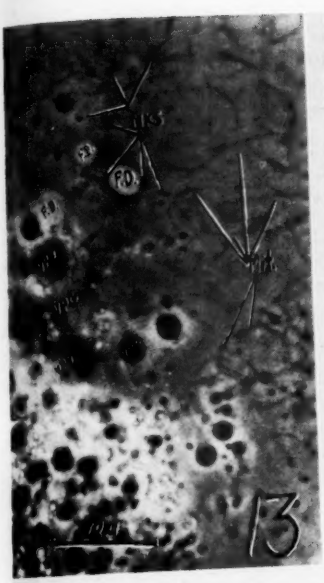
FIG. 13. The mitochondria (Mit.) and the *microkinetospheres* (MKS) are indicated in the cytoplasm of a *VP cell* (*Phase contrast*).

FIG. 14. *VP cell* of strain *HeLa* cultivated in a human serum nutrient which demonstrated *VP satellites* (SAT.) intimately associated with the *VP granules* (VPG). The nucleus is indicated by *N* and the nucleoli by *Ns* (*Phase contrast*).

FIGS. 15 and 16. Photographs of *VP cells* in the degenerating granular bag forms (*Phase contrast*).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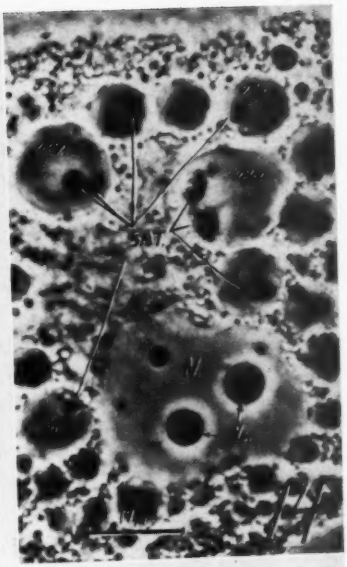

15

F.D.
F.D.
16

strated the *microkinetospheres* as the fastest moving component of the cytoplasm. The contacts which they made with the fluid droplets could easily be seen without the aid of the time-lapse camera.

The Variant Pinocytic (VP) Cell. A few hours after the basic nutrients were replaced with serum nutrients the *VP cells* commenced to manifest themselves in some cultures (Figs. 9–16). Their presence at this early interval was determined by the serum nutrient used and my the concentration of the serum nutrient. Cultures exposed to 100 per cent serum nutrients usually demonstrated *VP cells* before identical cultures in the same serum nutrient of a lesser concentration. From some patients 50 per cent serum nutrients produced *VP cells* in three hours whereas from other patients 100 per cent serum nutrients failed to stimulate their production in like cultures in the same interval of time. Since "major" pinocytes were practically limited to the periphery of these circular cell colonies it was here also that the *VP cell* became evident. Before the time-lapse studies were made, periodic observations of the cultures after the serum addition failed to disclose how the enlarged *VP granules* were forming. The cytoplasmic granules of early forming *VP cells* were only slightly larger than granules in cells bathed in the basic nutrients. They were presupposed to represent, then, merely a swelling of the already existent juxtanuclear granules. It was not until several hundred feet of 16 mm. film had been viewed that the true transformation was revealed.

In analyzing the film strips from the time-lapse sequences it was soon apparent that this phenomenon was truly a variant pinocytic process which differed sharply from that described by Lewis and later by Gey. The two pinocytic mechanisms paralleled up through point six but differed from point seven *(see summary)* in that the droplet did not reduce its size; nevertheless, it did shift its phase visual display (Figs. 10-11). This variation left a cell with large black spheroidal granules which mingled with the pre-existing lesser-sized granules of the juxtanuclear area. The phase shift was gradual and occurred over a varying length of time ranging between thirty minutes to several hours. During this phase shift an intermediate hue was reached which in the movies appeared as though the droplet had disappeared (Fig. 10). Eventually an intense blackening (B & L phase) occurred and

at this time the droplet became very round and rigid. During the intermediate color period the droplets often coalesced readily. This coalescence did not occur after the formation of the rigid ball-like granule. These *VP granules* ranged in size from 4-25 *micra*. The number of granules per cell varied with the pinocytic activity and the coalescence that occurred. Some cells actually had only one *VP granule* whereas the bulk of them had 25–100. Extreme numbers ranged up to several hundred. *VP granules* formed the first twenty-four hours were generally smaller than those formed the second twenty-four hours. A tremendous variation occurred among the cultures in the presence of different sera. One serum might stimulate *VP cell* production for two days whereas another for four or five days. Short lasting stimulation was followed by an epithelialization as in *pinocytosis* in the basic nutrients. In this instance the activity of the *VP cell* ceased with the epithelial surrounding. The *VP granules* slowly withered and disappeared from the cell. This withering occurred over a period of several days and though ill-defined appeared as a shrinking of the granule. Occasionally the granule appeared to collapse internally.

Where *VP cell* formation was prolonged (several days) the granules continued to fill the cells until a granular bag had developed (Figs. 15–16). These bags became a degenerate cell and fell away from the glass walls of the chambers. Granular bags were formed in twenty-four hours in an occasional serum and when this occurred the granules were of lesser proportions than those of *VP cells* of several days growth. Granules in these bag-like cells lost their severe blackness and roundness and became a closely packed and highly refractile facetted aggregate.

When weakly stimulating sera was re-added to a culture whose *VP* activity had subsided, a new response was seen. Occasionally a serum would be completely lethal to a culture and by twenty-four hours no viable cells could be found.

A 100 per cent serum nutrient would stimulate a production of *VP cells* which was two to six times greater than that of the same nutrient in a 50 per cent dilution with Earle's balanced salt solution. Similarly the 50 per cent serum nutrient provoked a response two to six times that of a 25 per cent nutrient from the same sera.

In some cultures each of the *VP granules* had a small satellite

granule (Fig. 14). The size of these *VP satellites* (less than 10 *micra*) was often directly proportionate to the size of the parent *VP granule*. At first this was thought to be merely a chance affiliation with the juxtanuclear granules normally present. Closer observation showed that these pairs of granules were intimately related and bonded inasmuch as they rotated and moved together rather than independently of each other. Generally speaking, if satellites were seen in one *VP cell* they were seen in all of the *VP cells* of that particular culture. Like the *VP granule*, the satellites were vitally stained with neutral red although the intensity was somewhat diminished.

The escorting duty of the tiny pre-existing juxtanuclear granules *(microkinetospheres)* (Fig. 13) mentioned previously in the description of *pinocytosis* in the basic nutients was realized first while studying film strips of the *VP cells*. This was a constant feature in both the pinocytic and variant pinocytic cells and as such must have been an intimate feature of *pinocytosis* which heretofore had escaped evaluation.

When equivalent sized cultures were exposed to serum nutrients from the same patient a similar *VP* response resulted. If one of a group of replicate cultures demonstrated 50 *VP cells* in a twenty-four hour period, so did the other cultures demonstrate similar figures. These values were not the same but as an instance were 50, 44, 41, 59. Another group of four equivalent-sized cultures exposed to another patient's serum might demonstrate in twenty-four hours 99, 109, 115, and 88 *VP cells*.

VP cells were stained most successfully with neutral red (Koehring, 1930). After returning the culture to its serum the comparison between the neutral red staining and the index of refraction shifting was made. These appeared to coincide precisely. The entering fluid droplets were colorless but as they moved into the cytoplasm and were contacted by the *microkinetospheres* the droplet commenced to take on the red coloring as well as display a contrast reversal. The *microkinetospheres* were not observed to change their color under bright field illumination in the presence of the neutral red.

Discussion

From the information now at hand we can say that *pinocytosis* was an expression of some cells to an environmental condition.

This expression must have been a physiological reaction to one or more stimuli. The question arose whether the stimulating feature of the nutrient produced *pinocytosis* as a stress reaction which tended to eliminate a noxious agent from the fresh media.

Role of Pinocytosis. Originally Lewis compared *pinocytosis* with *phagocytosis*. Although the role of *phagocytosis* has been thought of as one of cellular ingestion of nutrients, at times it probably has been an expendable function. Leucocytic cells from inflammatory areas have been frequently so stuffed with bacteria and cellular debris that they have succumbed by virtue of their great voraciousness. In this case, *phagocytosis* though a digestive function became a degenerative one so far as this particular phagocyte was concerned. Lewis did not think of *pinocytosis* as a degenerative phenomenon but as one which was purely digestive. From the information presented in this report and in light of the similarity between *phagocytosis* and *pinocytosis* a new interpretation on this important function has been passed. Though *pinocytosis* was probably a digestive action it was also probably one associated with a stress environment. In other words, "major" *pinocytosis* resulted in cells which found the environment undesirable at a particular time and through the cells' digestive function of pinocytosis attempted to eliminate the offending factors. This reasoning was substantiated by the following: (1) *pinocytosis* was a prominent feature of the cultures following fresh nutrient additions but not of reused ones, (2) mitosis and cellular multiplication was at a high level as the *pinocytosis* subsided, (3) the production of the *VP cells* after serum nutrient additions varied with the donor patient and was substantially equivalent in replicate cultures, (4) the *VP cell* was an expendable cell which succumbed in many instances as a granular bag after maximally engorging itself, (5) some cultures showed *VP cells* which developed to a marked degree and then suddenly ceased this activity. New cells emigrated about these *VP cells*, the granules of which eventually resolved so that within a few days the cell was not distinguishable as a *VP cell*. This last activity seemed to bear out the thought that the *VP cells* were removing an undesirable agent in the nutrient and at least in some instances they were successful. In further support it was shown that a large culture with one serum nutrient demonstrated its maximal number of *VP cells* in a shorter period of time than a smaller culture in the

same serum. This indicated that the offending agent was removed and the unrestrained growth was resumed earlier in the large culture which had more potential pinocytes than the smaller. Generally speaking, nutrients, whether of serum or serous cavity origin, were most growth promoting when the least amount of pinocytosis was seen. Recently, studies with strain *HeLa* in the synthetic media of Healy, Fisher and Parker (1955 (Connaught Medical Research Laboratories, Medium No. 1066)) have further borne out the suggestion that pinocytosis was associated with a stress environment. Cultures in Medium No. 1066 which contained supposedly ideal components displayed very little *pinocytosis* relative to that seen in the other basic nutrients but did show early epithelialization and abundant growth. Here, then, cells grew rapidly without the usual pinocytic features and indicated that pinocytosis was not always a necessary activity which prevailed before this enhanced epithelialization could occur. The evidence would tend to define *pinocytosis* as: (1) an intracellular digestive mechanism which eliminated from the fluid environment factors which restrained cellular regeneration and (2) a mechanism which if provoked excessively resulted in death of the pinocyte: hence, a degenerative phenomenon.

A point in regard to the emerging fluid droplets was the manner in which they were frequently projected into the cytoplasm. This occurred as though the droplet penetrated an invisible barrier, a secondary membrane or simply crossed from a dense to a less dense medium (gel to sol). Plasmagels at the periphery of the cell and plasmasols centrally were discussed by De Robertis *et al.* (1954). These authors referred to *thixotrophy*, a phenomenon of reversible solation-gelation and *syneresis*, a process of gel contraction which expelled a part of the liquid phase of the gel along with the dissolved substances. The undulating, waving and cupping motions of the pinocytic cell membranes may well have been an expression of *thixotrophy* and *syneresis* of gels in the periphery of the cell.

Interplay of Microkinetospheres. Without a doubt the interplay between the *microkinetospheres* and the incoming fluid droplets was an integral part of the *pinocytosis* seen in both cultures which demonstrated the usual pinocytic features and those which demonstrated *VP cells*. These *microkinetospheres* (0.3–0.6 micra) were by far the most active component of the

cell viewed. Without time-lapse studies but with the naked eye through the phase-contrast microscope these bodies moved in a deliberate manner which was at times very slow and at times quite rapid. With the time-lapse movies this movement was often so exaggerated that it was difficult to follow their courses. Lewis pointed out that the fluid droplet probably received an enzyme which caused it to shrink. This idea was developed because the shrinking droplet took up neutral red, a dye known to me an indicator of trypsin, erepsin and pepsin. After the contacts were made between the *microkinesospheres* and the fluid droplets the neutral red reaction occurred. The *microkinetospheres* were possibly the enzyme carrier which discharged some digestive component into the droplet and then moved on to other droplets. These *microkinetospheres* were too small to conclusively determine whether they dyed with either neutral red or Janus green. The impression was that they did not combine with either dye.

The VP Cell. When the *VP cell* was first recognized as an expression of human serum nutrients it immediately assumed a position of potential dignostic qualities. The fact that this cell was not noted in *HeLa* cultures bathed in basic nutrients obtained from ascitic and pleural effusions but did dramatically appear in cultures which were shifted to nutrients of human sera, automatically brought forth an anticipation that something unusual was created rather than found. The *HeLa* cell in serum nutrients altered its activities to meet the environment. In other words, the *VP cell* was probably a microscopic demonstration of the wonderful ability cells possess to adapt themselves and, hence, a whole organ or host to the environment. This appeared to be a blocking phenomenon which occurred after the *microkinetospheres* had contacted the fluid droplets. The fact that the droplets slowly shifted their index of refraction as indicated by the phase change from white to black at the same time they acquired a pink hue with the neutral red possibly indicated that a digestive enzyme entered the now forming *VP granules*. These droplets in the *VP cells* did not shrink though they continued to darken (phase contrast) and become redder (neutral red, bright field). If *pinocytosis* was to be thought of as a digestive function then the fluid droplet was not being digested, at least, at the same time as those seen in pinocytes in the basic nutrients. As the droplet became intensely black (phase) it also appeared to become a per-

fect sphere with an apparent hard coating. This hardness was assumed because the *VP granules* bounced off each other when observed with the time-lapse movies. The fact that most sera produced a similar number of *VP cells* in similar-sized cultures over an equivalent length of time and that these values often showed extreme variation among the different groups of donors did point out, as originally assumed, a potential diagnostic value of the *VP cells*. The satellites seen in some cultures were a recent finding. Their precise role in the *VP cell* has not been fully determined.

Although the emphasis of this discussion has been placed on the defensive position of *pinocytosis* it could not be said unequivocally that this was its major function. The unrestricted growth period which followed pinocytic waves could have been the result of a nutritious digestive phase. The evidence accumulated appeared to favor the former supposition inasmuch as a prolonged pinocytic period continued into a granular bag and cell death phase which eventually destroyed entire cultures. With the time-lapse-cine techniques almost every cell with a free membrane border showed a ruffling and this was more than likely a submicroscopic cell drinking or *potocytosis* which appeared to be a usual cellular phenomenon. We can now adjust our thinking to the concept that *potocytosis* was a normal cellular digestive mechanism and that *pinocytosis* was its accentuated counterpart which became visible with the light microscope. This accentuated form of fluid ingestion occasionally was seen to pass the limits of normality and beyond this point became the expendable fluid scavenger which resulted in the granular-bag forms.

Forthcoming experiments will attempt to refine our understanding of the *microkinetospheres*, the *satellites* and many of the points which are still within the realm of possibilities and suppositions. One of the most challenging prospects in this area of investigation is *selective pinocytosis*. A whole new field of therapeusis might unfold if a malignant cell could be induced to ingest a fluid medium ahead of the normal cells, to have its normal *potocytosis* inhibited, to select a lethal component (antimetabolite) from the fluid medium or to be pinocytically altered in some detrimental manner. If the *VP cell* or some other serologically promoted cell of tissue cultures could define its stimulating nutrient as being from normal or cancerous hosts, a diagnostic

method would be conceivable. These approaches are now being considered and investigated as the sequel to the data presented herein.

Summary

The pinocytic process as observed in strain *HeLa* cells which were cultivated in ascitic and pleural fluids followed these events: (1) a fanning and cupping activity of the cell membrane, (2) an engulfment of fluid droplets, (3) a rapid coalescence of these droplets immediately following their admittance into the cell, (4) an apparent sudden release of some of the coalesced droplets into the cytoplasm, (5) a contact of the white fluid droplet by black minute granules *(microkinetospheres)* which advanced from the juxtanuclear area, (6) a mitochondrial interplay of undetermined and inconstant nature, (7) a rapid rounding and shrinking of the incoming droplet, (8) a further coalescence of neighboring droplets, (9) a rapid advancement across the cytoplasm to the nucleus and juxtanuclear zone and finally (10) a shift in the phase visual display to black as the now shrunken droplet disappeared in the juxtanuclear zone as a minute granule.

To recapitulate the *VP* phenomenon, a list similar to that enumerated for pinocytosis in the basic nutrients follows: (1) a fanning and cupping activity of the cell membrane, (2) an engulfment of fluid droplets, (3) a rapid coalescence of these droplets immediately following their admittance into the cell, (4) an apparent sudden release of some of the coalesced droplets into the cytoplasm, (5) a contact of the white fluid droplet by black minute granules *(microkinetospheres)* which advanced from the juxtanuclear area, (6) a mitochondrial interplay of an undetermined and inconstant nature, (7) a shift in the index of refraction as indicated by a contrast reversal *without* any droplet shrinkage to a graying color, (8) a further coalition of droplets in this and earlier phases, (9) a rounding and intense blackening of the *VP granule* with no further coalescence, (10) a development in some cultures of *VP granule* satellites of a size proportionately smaller than the parent *VP granules* and finally (11) a collapse of the *VP granule* from a spherical rigid structure into either (a) a facetted and highly refractile component of a degenerating cell in a granular bag form or (b) a shrinking com-

ponent of a cell which eventually disposed of these granules by some form of dissolution.

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THE BRAINSTEM RETICULAR FORMATION

GLENN V. RUSSELL*

A widespread reawakening of interest on the part of anatomists, physiologists, and psychologists in the anatomy of the central reticular core of the brainstem has occurred in recent years. It has become evident that the cerebral cortex and corticothalamic mechanisms are in some way dependent upon this region of the central nervous system for the mediation of consciousness, attention, and integrated thought and action. No physician need be reminded that trauma or infection which involves these tegmental regions may result in altered states of consciousness and motor dysfunction.

The importance of the brainstem reticular formation in the integration of motor activity was recognized in the 19th century. A suggestion of an ascending influence upon the cortex was made early in the 20th century (4). Recently the concept of a "centrencephalic" system was introduced (11) to designate the hypothetical system of central, highest level neuronal integration which is presumed to occupy the upper brainstem tegmentum. The anatomical correlates of this system would appear to include the central core of brainstem reticular formation, the rostral continuation of this region into the basal diencephalon, and a corticipetal pathway from the diencephalon as a diffuse, non-specific thalamo-cortical projection system.

Recent reassessment of the cytoarchitecture of the reticular nuclei (9) substantiates, in general, the classical subdivision of this region into inferior, middle, tegmental, and superior reticular nuclei derived from the hindbrain; and a deep reticular nucleus of the midbrain. Evidence exists for some differences of function and connections of these two very similar regions.

The intrinsic fiber connections of the reticular nuclei remain poorly understood. The primitive pattern of short connections characteristic of the poorly differentiated central core of gray matter persists in these regions, and great ingenuity will be required to sort out the fundamental patterns of internal relationships. Development of successive suprasegmental integrative

levels and of more complex actions and behavior led to the development of long pathways within the central nervous system; however, their intimate association with the already extant multi-synaptic routes through the reticular formation was for many years not fully appreciated. Recent efforts have been directed towards assessing these extrinsic connections of the reticular nuclei, both afferent and efferent, and much fundamental anatomy is now known (Fig. I).

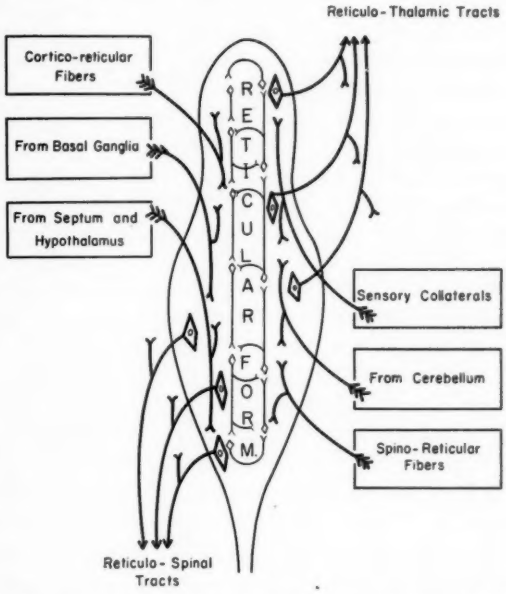

FIG. I. Schematic presentation of the fiber connections of the central reticular core of the brainstem.

The afferent fibers to the nuerons of the reticular nuclei come from almost all other regions of the central nervous system. Recently the greatest emphasis has been placed upon the numerous collateral branches of the ascending secondary sensory pathways (2, 13, 15). It may be presumed that all modalities of sensation contribute some innervation to the central reticular core of the brainstem. A direct spino-reticular system has been described (1, 6, 7). The cerebellum projects widely to the nuclei

of the reticular formation. The septal, preoptic, and amygdaloid regions contribute a significant descending system of fibers which is joined by fibers from the hypothalamus and passes into the reticular formation from above. A large portion of the outflow from the basal ganglia reaches the reticular nuclei of the midbrain and more caudal portions of the reticular formation. Indeed, for many years these paths from the basal ganglia were thought of as the principal afferents to this region. Lastly, the cerebral cortex gives an immense number of fibers into the reticular formation. These are both direct cortico-tegmental fibers and collaterals from other cortico-descending pathways. A heavy system of fibers enters the midbrain reticular formation from the parieto-occipito-temporal association cortex. A pathway from orbito-frontal cortex through the hypothalamus and into the tegmentum may be a fundamental link in the mechanisms of attentive behavior.

Efferent projections from the reticular nuclei may be separated into descending reticulo-spinal and ascending reticulo-diencephalic pathways. The former are well known (10) and are recognized as an integral part of the parapyramidal system. They play an important role in the regulation of muscle tone and posture. Particular regions of the more caudal reticular formation have been identified as "centers" which exert a regulatory influence over vital functions such as respiration, cardiovascular function, urinary continence, etc. These "centers" are connected with appropriate spinal cord levels by the reticulospinal tracts.

Until recently the existence and importance of ascending reticulo-diencephalic pathways had not been recognized. Neurophysiological observations, however, led to the proposal of such a system, and it was designated as the "ascending reticular activating system" of the brainstem (5). Two reticulo-thalamic fiber complexes have been demonstrated and suggested as the anatomical correlates of this system (12). Both terminate in the ventro-basal parts of the diencephalon, including dorsal, sub-, and hypothalamic regions. The terminations in the dorsal thalamus are largely in the intralaminar nuclei. The name *intralaminar* derives from the position of these nuclei in the internal medullary lamina of the thalamus. They have been interpreted

as a part of the rostral continuation of the brainstem reticular formation.

Intrinsic connections within this nuclear complex provide a rostrally coursing intrathalamic pathway which spreads into the nucleus reticularis of the thalamus and to the basal ganglia and perhaps to the cortex. Fibers also spread from the intralaminar nuclei into the other more familiar relay and association nuclei of the thalamus (8). The reticular nucleus with its widespread non-specific projections to the cerebral cortex forms the final link in the ascending reticulo-thalamo-cortical mechanism.

Demonstration that a stimulus delivered to the reticular formation alters the electrical activity of the cerebral cortex has led to an extensive series of researches upon the functional role of this region. It has been shown that a wide variety of sensory stimuli, as well as direct stimulation, are equally capable of evoking activity within the "ascending reticular activating system." This effect is relayed through the diencephalon and thence widely to the cerebral cortex of both hemispheres. The effect is described as an "activation of the EEG" or an "arousal" reaction. The bilaterality of the effect is best accounted for by our knowledge of well developed commissures which interconnect the midbrain reticular nuclei of the two sides.

In the hands of some investigators diversified sensory stimuli and direct stimulation in the course of the "ascending reticular activating system" and intralaminar regions of the thalamus have been considered equipotent insofar as the final observed effect they produce upon the EEG. These persons have thus been led to hypothecate a global or unitary functional capability for these structures. Such a concept may well account for consciousness as an integral phenomenon, but can hardly explain the equally important differential aspects of the conscious and attentive state. Therefore, others have been led to seek and to find differences in the response evoked in the cerebral cortex by diverse modes and loci of stimulation.

Several bits of anatomical evidence would support a view of multiple and specialized effects from various segments of the "centrencephalic" system. The wide areas of the central nervous system that project into the region, and the complex interconnections between part of the system and adjacent structures suggest specific actions as opposed to unitary generalized activity. Re-

cently it has been reported that stimulation in the reticular formation is able to influence directly the propagation of impulses in a sensory relay nucleus (3). We are all aware that attention to some particular task tends to "block" awareness of other sensations and mental processes unless the latter are of a form or magnitude to effectively "break into" our thoughts. Only as I now direct the reader's attention to them are you aware of the sense of contact with the chair or the pressure of clothing and shoes. The effective suppression of extraneous sensations and thoughts, like the effective suppression of excessive and undesirable motor activity, appears to be a function of the reciprocal relationships that exist between cerebral cortex and reticular formation. It can hardly be interpreted on the basis of a global hypothesis of reticular formation function.

It would seem that we have made great strides in our effort to unravel the problem of the mechanism of levels and states of consciousness. Two rather different actions of the reticular formation appear to be involved. First, a global or unitary effect appears to be exerted downwards upon the motor cells of the bulb and spinal cord and upwards through the diencephalic reticular formation upon the cerebral cortex. This effect serves to provide a background level of nervous activity observable as coma, deep sleep, light sleep, relaxed inattention, alert attentiveness, or hyperattentive emotional states. Each of these levels may be directly related to EEG patterns and to the general tonic state of the body musculature. Secondly, we may identify a specific localizing effect that also acts both downwards and upwards, and which mediates the more complex aspects of attentive phenomena.

I have attempted to summarize the state of our knowledge of anatomy that may be interpreted as the morphologic elements of consciousness and attention. Many questions lie unsolved, even unasked. They are now under concerted attack. A clear postulation of the function of the reticular formation will have to be based upon adequate and complete knowledge of the anatomy of the reticular formation.

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SURGICAL TREATMENT OF PERIPHERAL ARTERIAL DISEASE IN GERIATRIC PATIENTS*

A. O. Singleton, Jr., Maurice Adam, and Frank Dunton

In an unselected group of 92 geriatric patients with peripheral arterial disease treated surgically at The University of Texas Medical Branch Hospitals there were 49 amputations. This not only represents a failure of less heroic treatment but means a more incapacitated individual who becomes more susceptible to the other infirmities of old age. Admittedly, many of these had no treatment or improper medical management before reaching our institution. While many of these patients represent impoverished individuals and of lower mental status, well-to-do persons were likewise present. The great majority of these cases consisted of obliterative, arteriosclerotic vascular disease (63 patients), with rarer cases of embolism (8), and aneurysms (8). Thromboangiitis obliterans (11 cases) and angiospasm (2) without organic vessel changes, while primarily diseases of younger individuals, also are represented in this older age group. Operations for peripheral arterial disease falling short of amputation fall into two categories: lumbar sympathectomy in an attempt to improve collateral circulation and relieve any associated arterial spasm that might be present as a part of the pathological process, and attempts to remove or bypass the obstructive process. Peripheral neurectomy has been used as a palliative measure, but has been of questionable value.

In the past there have been many enthusiastic advocates of lumbar sympathectomy in arteriosclerotic vascular disease, but more recent work shows diminution in the enthusiasm for this procedure. Pratt (1955), reporting 309 lumbar sympathectomies for occlusive arterial disease, found that 11 per cent of the patients who had arteriosclerosis without diabetes came to amputation. In those with arteriosclerosis and diabetes, the rate was 34 per cent. In the thromboangiitis obliterans group, 20 per cent subsequently had amputations. Our figures for these groups were 13 per cent, 60 per cent and 33 per cent respectively, with an overall rate of 22 per cent. Where the sympathectomy in Pratt's

cases was done for coldness and claudication there was an amputation rate of 4.4 per cent. When ulceration and infection were present, 32 per cent required amputation with a 69 per cent amputation rate in the presence of actual gangrene. Those individuals, who had pain at rest, lost their limbs in half of the cases. Our amputation rate was 39 per cent where infection, ulceration or gangrene was present; 25 per cent without. Berry (1955) at Ann Arbor, in reviewing the results of 397 lumbar sympathectomies performed on 275 patients, during the 1945 to 1952 period, for arteriosclerosis obliterans of the lower extremities, felt that only 34 per cent of such patients were improved. Less than 50 per cent of our cases showed any improvement after sympathectomy. Poor results without amputation were observed in 20 per cent of Berry's cases, with an amputation rate of 41 per cent.

It is interesting to note that White, Flascher, and Drury (1955) could not demonstrate any increase in collateral circulation in the cat following lumbar sympathectomy three to four weeks after superficial femoral artery ligation above the already dilated vessels.

In 1956 Halligan, measuring circulatory efficiency with radioactive, iodinated serum albumin, reached the conclusion that sympathectomy should be limited to individuals with moderate arteriosclerotic vascular disease.

In 1956 Nelson and Tremble at Johns Hopkins University found that lumbar sympathectomy in their group of cases had proved an unsatisfactory therapeutic measure in from 59 to 74 per cent of cases of arterial occlusive disease. In their series of 192 patients, representing 272 extremities which underwent lumbar sympathectomy for various disorders, they felt that it was impossible to predict the result of operation for an individual patient by any clinical or laboratory procedure used for preoperative evaluation, including the presence or absence of pedal pulses and in the use of the skin resistance test. They felt that a more vigorous attempt be made to screen patients for possible direct attack on the offending occlusion in view of the poor results of sympathectomy.

Although Grimson (1955) has felt that excision of the lower thoracic and all of the lumbar ganglia have given satisfactory results in patients with obstruction of the terminal aorta or iliac

arteries with viable limb, most of the recent publications have stressed mechanically removing or bypassing localized obstructions of peripheral vessels.

There has been a great difference of opinion as to whether arterial endarterectomy or forming a new channel by means of grafts give the most satisfactory result. The utilization of these procedures depends on demonstrating by means of arteriography a more or less localized area of blockage of the vessel. Where occlusion has occurred distal to the popliteal, or where a generalized occlusion of the vessels of the extremity has occurred, these methods are not indicated. The most important factor is whether the outflow will equal the inflow after correction of the obstruction. Arteriography in itself is not without hazard.

An aortagram is usually indicated where evidences of ischemia are present in a lower extremity and the femoral pulse is not palpable. In experienced hands with good technique this is often sufficient to show the extent of a limited obstruction, as a patent vessel below the point of blockage may be visualized by collateral filling. In some cases it may be necessary to perform a femoral arteriogram or explore the femoral vessel in order to determine whether a lumen is present below the point of obliteration, as demonstrated in the following case. V. J., a 51 year old colored woman, had a necrosis of tissue over the right heel and absence of pulses in the leg. No major vessels were demonstrable in the aortagram. A femoral arteriogram supposedly demonstrated a patent vessel. On surgical exploration the right iliacs and femoral were found to have no lumen; the vessel visualized by arteriogram was the femoral profunda. A lumbar sympathectomy was done. Following this, pulses could be palpated at the ankle and it was possible to successfully cover the heel defect with a skin graft.

R. P., a 50 year old white man, represented the type of case where surgical treatment short of amputation is of no benefit to the patient. There were extensive calcifications of the vessels by X-ray. No femoral pulses were palpable but an aortagram showed that the vessels were still patent (Fig. 1). Despite a previous bilateral lumbar sympathectomy gangrene of the left big toe and part of the foot had occurred.

A more obvious localized block in the popliteal region was demonstrated (Fig. 2) but because of the presence of gangrene

in the foot, the absence of adequate vessels distal to the obstruction, and the general calcification of these vessels, an amputation was done with no attempt made to bypass the obstruction.

While resection of the stenotic area with bridging by a graft was originally popular, Crawford, De Bakey and others now feel that a bypassing procedure, which avoids destruction of collaterals in the region of the obstruction, is superior in obstructions below the iliac vessels.

The bridging or bypassing channels have most commonly been formed by arterial homografts with good results reported in most cases. Shaw 1955 reported improvement in 16 out of 22 extremities so treated. Linton (1955) reported good results in 77 per cent of cases. Arterial homografts have been more successful than autogenous or homologous vein grafts. Homografts have been preserved in a number of ways, including the nutrient broth method, the quick freeze method, the freeze dry method, and the frozen irradiated method. In our own hands, preservation of homografts in 70 per cent alcohol without freezing or refrigeration has proven to be satisfactory.

Many grafts have been made from snythetic materials such as Nylon, Vinyon-n, Orlon and Dacron, the chief value of these grafts being their accessibility at all times and in all localities. Only long term use will determine how successful these substitutes will be. McAllister (1955) replaced segments of the femoral or popliteal arteries in 10 instances with tubes of synthetic fabric with success in 7 cases, thrombosis occurring in three.

Despite many brilliant results reported in the use of vessel grafting, there have been discouraging reports. Hoye (1956) found in follow-up studies of attempts at arterial reconstruction by endarterectomy and by grafts in 29 patients, that 70 per cent of 31 vessels had become closed over a 2½ year period. From the results of experimental work, questions have been raised as to whether aneurysms might not arise later at the site of the grafts.

Though fewer in numbers, good results have been reported following thromboendarterectomy. Coelho, Leeds and Freeman (1955) have reported full recovery from impaired circulation of the lower extremities in 18 out of 20 cases with terminal aortic or iliac occulsion followed from one to 39 months.

Cannon and Barker (1955), in treating selected cases of ar-

teriosclerotic occlusive disease of the superficial femoral and popliteal arteries, reported good results in 20 out of 23 cases whose popliteal tree was demonstrated to be patent before endarterectomy. No incidence of thrombosis in the endarterectomized segment of an artery in which a pulse was restored has been encountered in a follow-up period extending as long as 35 months.

T. B., a 45 year old colored man seen at our institution, had intermittent claudication and pain in the left hip and leg brought on by walking. No pulses were palpable in the femoral region or distally. He was asymptomatic in the right leg. An aortogram showed an obliteration of both external iliac vessels and the internal iliac on the left (Fig. 3). Femoral arteriograms were then attempted by surgical exposure of the vessels. The right femoral lumen was found to be obliterated. The left was found to be patent up to the inguinal area. An endarterectomy was performed on the occluded left external iliac and upper femoral artery together with left lumbar sympathectomy. The patient was improved symptomatically with a palpable femoral pulse but pulses at the ankle were never elicited. An aortogram performed three months later showed an area of obstruction present in the upper femoral area, previously felt to be patent (Fig. 4). A bypassing of the obstructed area with a graft was planned but the patient refused further surgery.

T. H., a 45 year old white man with intermittent claudication, had a left common iliac obstruction with patent external iliac demonstrated by arteriogram. This was treated by endarterectomy and left lumbar sympathectomy, with improvement of symptoms of vascular ischemia though pulses at the ankle were not demonstrated. The patient has been satisfied with the result

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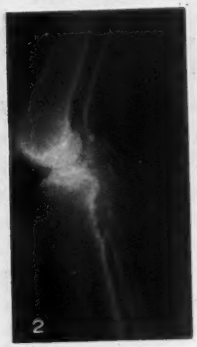
FIG. 1. Aortogram showing patency of iliac and femoral vessels, although no femoral pulses were palpable. (Patient A.P.)

FIG. 2. Arteriogram demonstrating localized block of popliteal artery. Patient R.P.)

FIG. 3. Aortogram demonstrating obliteration of both external iliac vessels and the internal iliac on the left. (Patient T.B.)

FIG. 4. Aortogram of same patient shown in Fig. 3 performed three months later following endarterectomy of left external iliac and upper femoral artery. The external iliac vessel on the left is now patent but there is a localized blockage of the upper femoral vessel. The lower left femoral vessel has been filled by the opaque media passing through collateral channels.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to the point that he has not wanted further surgery attempted though he tires on walking more than three blocks.

T. G., a 75 year old white man, had a femoral aneurysm involving the bifurcation of the common femoral artery. After resecting the aneurysm, a pants-like dacron graft was used to bridge the defect. His pulses remained strong and the circulation good following the procedure for about six months when the graft apparently thrombosed. The area was explored when the patient returned one month later with pain in the leg and evidence of poor circulation. On re-exploration it was found that thrombosis had extended up to the external iliac. Because the patient was doing extremely poorly under anesthesia, a left lumbar sympathectomy was done but no attempt made to graft around the area of obstruction. Although, by all tests, the sympathectomy was successful, the circulation was not appreciably improved. The question of amputation versus an attempt to reestablish the circulation by means of a graft was discussed with the patient but he elected to have the amputation. In view of his age, his poor general condition, and the desire to have the local problem cleared up definitely as soon as possible, all convinced us that this was probably the wisest decision in this case.

In the surgical correction of an aneurysm in the larger vessels of an extremity, we believe that it is highly desirable to restore the normal arterial flow by grafts if repair of the vessel can not be made by end to end suture. This is especially important in the older individual whose circulatory efficiency is usually diminished. One must not only think of saving the extremity but of leaving a limb capable of maximum function.

In our eight cases of embolic occulsion involving five femoral and three popliteal arteries, good results following embolectomy occurred in three cases seen within 12 hours. The results were poor in the other cases, who entered the hospital three days or later following the embolus. These cases serve to emphasize the importance of early recognition and restoration of the arterial flow in these patients.

McKittrick (1949) and others, after allowing infection and ischemic necrosis to stabilize for many days or weeks under the influence of antibiotics prior to performing amputation, have been able to use trans-metatarsal and even lower leg amputations for diabetics in arteriosclerotic gangrene that were generally

considered unsuccessful before the era of antibiotics. Warren, Thayer, Achenbach, and Kendall (1955) have also applied this principle in selected cases in doing a Syme amputation. They report six Syme amputations for ischemic lesions of the foot with good results. However, when they extended this to five additional cases, where less conservative indications were used, two of these failed and the patient had to have higher amputations.

Our amputations were for the most part supracondylar with primary healing in 91 per cent of cases. The few amputations done below the knee and lower, with the exception of local removal of toes, either had to be re-amputated or had delayed wound healing in most cases.

Summary

In an unselected group of geriatric patients with peripheral arterial disease studied at The University of Texas Medical Branch Hospitals, 53 per cent came to major amputations. In 45 cases of arteriosclerosis treated by lumbar sympathectomy, 50 per cent were un-improved and 22 per cent came to amputation. In view of these poor results, it is felt all cases where localized areas of obstruction can be demonstrated should have an attempt made to remove or bypass the obstruction. As to whether such an approach will greatly alter the overall bad outlook in these patients remains to be shown, but marked improvement in individual cases has already been demonstrated.

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SYNCHRONOUS MITOSIS IN A CARDIAC INFARCT

JOHN G. SINCLAIR[1]

Mitosis is quite commonly regional but seldom synchronous. A discussion of initiating factors in mitosis will follow the presentation of a case of synchrony.

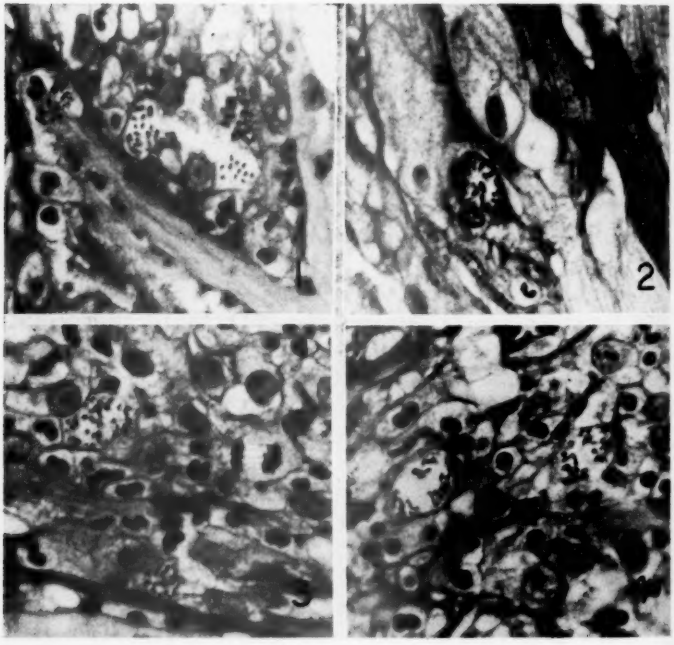
A series of experimental infarcts in dogs was produced by Dr. Arthur Ruskin by ligating the left coronary artery. Dogs were sacrificed at intervals from two days to twenty-two days. The author studied blocks from the infarcted areas. The sequence of events, while not uniform for the whole series, is about as follows: (1) The muscle fibers swell and obliterate the capillary spaces. Striations become very poorly defined and telophragma are very pronounced. (2) In patchy areas the fibers shrink, nuclei become pyknotic and may be ejected. (3) After several days, in subendocardial and subepicardial areas, infiltration of leucocytes occurs with sharply demarcated fronts among shrunken fibers. (4) Fibers lose their myosin and become pale skeletons of actin. (5) After hyalinization (6–10 days) the fibers dissolve while a gelatinous matrix of invading fibroblasts and leucocytes fills the space. (6) Mitosis increases the number of fibroblasts. (7) The leucocytes disintegrate and diminish while collagenous fibers are laid down in lines continuous with the functional muscle fibers.

The ten-day specimen contains areas, separated by several millimeters of unaltered muscle (Fig. 6), in which the matrix of stage 5 has been reached and stage 6 has started. Approximately one fibroblast in two hundred is in late prophase (Figs. 1–4). In examining about 1000 mitoses two exceptions have been seen. One is probably a smooth muscle cell in a small blood vessel left in the area and the other is an anaphase division (Figs. 2–3). Chromosomes are double and well separated. The diameter of the prophase cells is about twice that of surrounding cells. This varies in range as Lewis (11) described it for tissue cultures. The area is avascular. Leucocytes although pyknotic have not disintegrated. There are no mitoses outside of these areas.

Discussion

The intrinsic nature of the cause of mitosis is demonstrated by the fact that only one type of cell is involved and that only one in two hundred is affected. There are no intergrades to the leucocytes, which Allgower (2) intimates might be the source of fibroblasts. The smooth muscle which is present in abundance and well preserved is not stimulated though the cells are swollen. Only particular cells by internal accumulation of nuclear and cytoplasmic constituents are prepared to be triggered by external conditions.

Child (6) in 1940 and earlier, has noted that there are many types of regeneration and reorganization which are accomplished



FIGS. 1–4. Four examples of mitosis in an experimental cardiac infarct.
1. Three apparently confluent cells in prophase mitosis.
2. Mitosis among marginal muscle fibers. Prometaphase.
3. Prophase and anaphase in a cardiac infarct. Leucocytic area.
4. Cluster of mitoses in the leucoytic area.

by cell movements before increase of mitosis is seen. See also Medawar (13). This is certainly true of epithelialization of wound surfaces (Arey, 3) and we have seen it clearly in both epithelium and submucosal tissues in colonic anastomosis (Poth, *et al.*, 14). A certain amount of cell aggregation gives the optimum basis for proliferation in tissue cultures. In the cardiac infarct both migration by fibroblasts and infiltration by leucocytes is slow. The invaded area is avascular and presumably has a low oxygen tension. Fibroblasts can exist under conditions of oxygen deprivation which would be detrimental to all other cells. Cell division may be regarded as an escape from unfavorable conditions by increase of all surface-volume ratios.

The optimum conditions for mitosis seem to be delimited between the best and the worst oxygenation. These may be met in a stratified epithelium in the basal layer or may be shifted by controlled conditions to the middle layers of the stratum germinativum (Cowdry, 7). In young cartilage, mitosis occurs in a zone parallel to perichondrium but internal to it (Fig. 5).

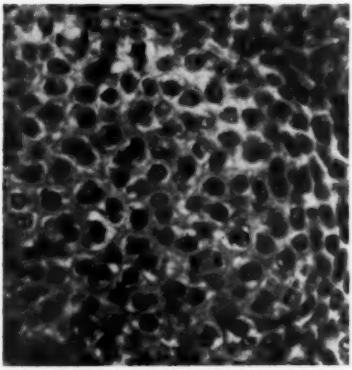


FIG. 5. Three anaphase mitoses in an embryonic cartilage showing the general zone in which it occurs.

This differs in the different cartilages of one embryo. Byerly (5) said that recovery after temporary suffocation produced a disordered mitotic flare in chick embryos. Recovery from urethane inhibition both in mammalian embryos and hemopoietic tissues of adults results in delayed acceleration of mitosis. Lymphomas have been produced by these means (Sinclair, 15, 16).

Specific stimulating substances as seen in artificial parthenogenesis seem to be based on their ability to coagulate cytoplasm and to release calcium ion (Heilbrunn, 10). The products of disintegration of leucocytes, muscle and blood stimulate wound healing. Baker (4) found glutathione to be essential to mitosis. Hammett (8) selected SH ion as the efficient factor, and Mazia

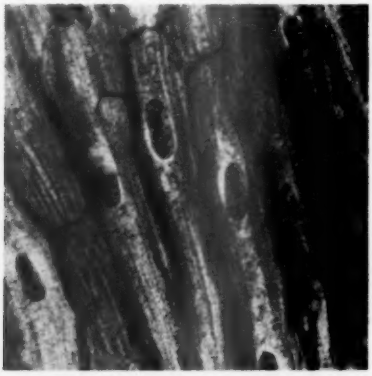


FIG. 6. Swollen muscle fibers of the cardiac infarct showing lack of infiltration, no mitosis, marked telophragma, actin fibrillae.

(12) located the position of sulphur as the binding force in the mitotic spindle. Precisely what is accomplished by shaving the abdomen of mice as Hance (9) did, or putting slight traction on feather follicles as practiced by Japanese chicken fanciers, is difficult to determine. In each case accelerated mitosis occurs some days after the manipulation. The diurnal rhythms seen in many mammalian tissues have been related to temperature cycles in which nocturnal forms are obverse to daylight active forms. The biochemical cycles of the serum would seem to be more probable factors. An extreme example of this cycle is seen in the glands of an acute appendix taken at 10:30 P.M. Each section of each gland shows from one to four mitoses. The mitoses in any one gland tend to be synchronous (Fig. 7).

Among the specific substances necessary for mitosis is desoxyribose nucleic acid. In blastulation the nuclear volume increases at the expense of cytoplasm without any intake. Here mitosis is at its highest known rate. Nerve cells, on the other hand, have plenty of DNA but never divide so it cannot be a critical factor.

In the cardiac infarct, fibroblasts could grow at the expense of leucocytes.

The synchronous mitosis of thousands of cells in the cardiac infarct indicates the presence of some factor that suddenly rose to a critical value. Such a factor is not identified. Within binucleate and multinucleate cells synchrony is the rule. An interesting case of intercommunication of three cells is seen in figure 1. Fluids which have bathed mitotic tissues are a stimulus to others in tissue culture. Embryonic serum and that of pregnant women is a better medium than other sera. The cyclic waves of mitosis in seminiferous tubules provide another variant of synchrony. The rather sudden final development of an ovarian follicle is another. These are incited by general hormonal influences acting on prepared trigger mechanisms.

FIG. 7. Section of a gland from an appendix removed at 10:30 P.M. The mitotic index is very high in every gland.

Summary

A rare case of synchronous mitosis in thousands of fibroblasts in an experimental cardiac infarct is described. All mitotic cells are in late prophase. The conditions which favor the onset of mitosis are discussed and illustrated.

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CALLING ATTENTION TO

Items of Possible Interest to Medicos

December, 1956

Can Hungarian Science Survive? Let's help: P Braun & Co give free amino acid content of lymph (*Acta Med Acad Sci Hungaricae* 10/67/56). M Foldi & Co study effects of hypoxia on kidney function in men (*ibid* 43). D Hattyasy & Co find seasonal shifts in flourine content of saliva in caries-resistant (gypsy) people (*ibid* 171). G Szabo & Z Magyar note effects of histamine on capillary permeability (*ibid* 55). F Vandor & S Boros discuss radionecrosis of mandible (*ibid* 145). D Schuler & K Balog find pneumonia big factor in newborn mortality (*Acta Morph Acad Sci Hungaricae* 7/35/56). L Vaczy shows cystic glandular hyperplasia predisposes to cancer of uterus (*ibid* 81). L Eidus & J Nuridsany find isonicotinic hydrazide causes ascorbic acid depletion of adrenals (*Acta Physiol Acad Sci Hungaricae* 10/101/56). E Grastyan & Co show conditioned alimentary & definsive reflexes can be sp facilitate or inhibited by electrical stim of sp points in hypothalamus & reticular formation (*ibid* 9/132/56). P Kertai & Co show intestinal absorption of various substances (glucose, NaCl, urea) involves energy consumption through ATP (*ibid* 10/33/56). J Knoll continues study of active focus in orientative searching in performance of motor function in conditioned reflexes (*ibid* 89). K Lissak & Endroczi find nerve tissue inhibitor of nerve conduction & chemical mediators (*ibid* 9/111/56).

Year-end Notes: RH Berg well discusses "medical ethics" (*Look* Dec/15/56, pp 69–72). DB Bonsal cheers group making *Report of the Sp Com on the Federal Loyalty-Security Program of the Asso of the Bar of the City of NY* (Dodd-Mead, NY, 56, 327pp, $5). C Bramwell discourses (NB AMA politicos:) on practice, teaching & research (*Lancet* 2/793/Oct 20/56). HD Fabing makes neurochemical inquiry into going "berserk" (*Sci Month* 83/232/56). P Klopsteg well considers university responsibilities, gov't money, & tax programs (*Science* 124/919, 965/Nov 9, 16/56). RE Lapp keenly comments on *Atoms & People* (Harper, NY, 56, 304pp, $4). R Linton examines *Culture & Mental Disorders* (CC Thomas, Springfield, Ill, 56, 152pp $6.5). VR Potter suggests support for study & reflection (*Cancer Res* 16/725/56). JM Badzinski indicates Russian practice of phrenophagia,—artificial human selection (*Am J Psychiat* 113/312/56). WH Thorp reviews *Learning & Instinct in Animals* (Harvard Univ. Press, Cambridge, Mass., 56, 367pp, $10).

Tooting Our Own: JC Kraner & E Ogden note ventricular suction (*Circ Res* 4/724/56). JM Ryan, RW Stacy & RN Watman show pulse wave contours alter on occlusion due to increase in pulse wave velocities & arterial pressures (*ibid* 676). LA Sapirstein finds 60″ cardiac output distribution by K^{42} as 18% kidney, 6% liver, 3% heart & 0.3% brain (*ibid* 689). EE Selkurt & GA Brecher note hemorrhagic hypotension impairs oxygen utilization in liver (*ibid* 693).

Clinical: RN Cooley & RD Sloan describe *Radiology of the Heart & Great Vessels* (Wms & Wilkins, Balt., 56, 30pp, $12). TE Friedemann edits symposium on nutritional & toxic aspects of irradiated foods (*Fed Proc* 15/905–937/56). JW Gofman edits report on serum lipoprotein & cholesterol estimations as predictors of complications of athersclerosis (*Circ* pt 2/691–742/56). RE Pattle & H Cullumbine find atmospheric sulphur pollution toxic (*Brit Med J* 2/913/Oct 21/56). NW Shock edits symposium on biology of aging (*Fed Proc* 15/938–964/56). MB Visscher & Co discuss lung edema (*Pharmacol Rev* 8/389/56).

Muscle Physiology: F Buchtal & Co survey chemical events in muscle contraction (*Physiol Rev* 36/503/56). HE Huxley reviews knowledge of muscle contraction (*Endeavor* 15/177/56). A Szent-Gyorgi discusses bioenergetics in muscle contraction & suggests quantum biology (*Science* 124/873/Nov 2/56).

Preclinical: M Alexander indicates uniform intracellular distribution of enzymes in microbial cells (*Bact Rev* 20/67/56). HJ Bein discusses pharmacology of Rauwolfia (*Pharmacol Rev* 8/435/56). WH Bridger & WH Gantt describe effects of mescaline on differentiated conditioned reflexes (*AM J Psychiat* 113/352/56). SW Fox & Co propose biochemical origins of optical isomers (*Science* 124/923/Nov 9/56). OH Gaebler edits Henry Ford Hosp symposium on *Enzymes: Units of Biological Structure & Function* (Academic Press, NY, 56, 624pp, $12). WD Gray & C Sova discuss biochemorphology of alcohols on glucose utilization by yeast (J Bact 72/349/56). RG Macfarlane surveys blood coagulation (*Physiol Rev* 36/479/56). G Pincus & Co note progestin action of 19-norsteroids (*Science* 124/890/Nov. 2/56). HW Smith gives *Principles of Renal Physiology* (Oxford Univ Press, NY, 56, 237pp, $7). H Veldstra surveys synergism & potentiation of drugs (*Pharmacol Rev* 8/339/56). WF Windle & Guth describe nerve regeneration (*Physiol Rev* 36/427, 441/56).

JANUARY, 1957

Hungarians Carry On: A Arvay & J Nagy show diurnal changes in uterine motility (*Acta Physiol Acad Sci Hungaricae* 10/199/56). J Biro & Co find ischemic shock inhibits conditioned reflexes with gradual restoration (*ibid* p 277). L Harsing & Co note ischemia kidney injury is reduced by hypothermia (*ibid* p 429). B Issekutz & Co indicate oxidative phosphorylation is lowered in muscle by denervation (*ibid* p 328). L Kadas & Zsanbeky recommend cortisone as adjunct to BAL to promote kidney regeneration in $HgCl_2$ poisoning (*Acta Med Acad Sci Hung* 9/363/56). S Simon finds dextran is more lasting plasma column expander than polyvinylpyrrclidone (*Acta Physiol Acad Sci Hung* 10/367/56). J Sos & Co show p-nitro-benzoyl-glutamic acid is metabolic antagonist of glutamic acid on micro-organisms & on brain function in rats (*ibid* p 407).

Tooting Our Own: FR Aumann discusses *The Instrumentalities of Justice: Their Forms, Functions & Limitations* (Ohio State Univ Press, Columbus, 1956, 137pp, $3). ER Caley & JFC Richards offer *Theophrastus on Stones*, with translation & commentary (Ohio State Univ Press, Columbus, 1956, 235pp, $4).

Brain & Behavior: PH Hoch edits centennary symposium on Sigmund Freud

(1856–1939) (*Bull NY Acad Med* 32/857–922/56). E Kahn surveys work of Emil Kraepelin (1856–1926) (*Amer J Psychiat* 113/289/56). S Levine & Co note effects of early shock on later avoidance learning (*J Personal* 24/475; 25/70/56). DG Mandelbaum edits E Sapir's essays on *Culture, Language & Personality* (Univ California Press, Berkeley, 1956, 224pp, $1.5). GH Mowbray reflects on man as a link in complex machine systems (*Sci Month* 83/269/56). CR Rogers & BF Skinner discuss issues concerning control of human behavior (*Science* 124/1055/Nov 30/56). CW Simon & WH Emmons review relations of EEG patterns to sleep & wakefulness (*ibid* p 1057). L Stein indicates reserpine does not inhibit learning of fear (*ibid* p 1082).

General: C Auerbach (discoverer of mutagenic action of mustards) discusses *Genetics in the Atomic Age* (Essential Books, Fairlawn, NJ, 1956, 113pp, $2). L Bartalanffy & A Rapoport edit *General Systems* (Yearbook Soc Adv Gen Systems Theory, Vol 1, Braunfield, Ann Arbor, Michigan, 1956, 310pp, $7.5). IB Cohen examines the philosophical relations of *Franklin & Newton* (Amer. Philosoph. Soc., Phila, 1956, 683pp, $6). M Grotjahr goes *Beyond Laughter* (McGraw-Hill, NY, 1956, 304pp, $6). G Hevesey traces the path of atoms through generations (*Sci Month* 83/238/56). HJ Muller expounds genetic principles in human populations (*ibid* p 277). P Tower reviews ophthalmological pioneering of George Bartisch (1535–1607) (*Arch Ophthal* 56/57/56). For an account of a big job see DH Wenrich story of *Biol Abts* (30 (7): 1805–1821, 1956). WH Whyte's *The Organization Man* looks like a must for non-conformists (Simon & Schuster, NY, 429pp, $5).

Clinical: JH Ebbs recommends elimination of glutinous foods in coeliac disease (*Canadian Med Asso J* 75/885/56). KH Fischer & BM Dlin conclude that potency of placebos derive from "emotionally invested doctor-patient relationship" (*Amer J Med Sci* 232/504/56). T Funaki discusses pathogenesis of Aschoff bodies (*Mie Med J* 6/129/56). A Gellhorn edits symposium on Hormones & Cancer Therapy (*Amer J Med* 21/657–759/56). M Gleason, Rx Gosselin & HC Hodges survey *Clinical Toxicology of Commercial Products* (Wms & Wilkins, Balt., 1957, 452pp, $12.5). W Loffler edits *Erkrankungen der Atmungsorgane* (Lange & Springer, Berlin, 1956, 4 vols, 4400pp, DM 583). TA Longmore describes *Medical Photography: Radiographic & Clinical* (Amer Photo-Book Publ Co, NY, 1956, 992pp, $15). W Stich edits *Insulin & Insulin Therapie* (Urban & Schwarzenburg, Munich, 1956, 178pp, DM 24). JH Talbot & RH Ferrandis discuss *Collagen Diseases* (Grune & Stratton, NY, 1956, 256pp, $6.5).

NB: American issue of IP Pavlov's *Experimental Psychology* (Philosophical Library, NY, 1957, 654pp, $6) seems like reprint of English translation of *Selected Works of IP Pavlov* (Foreign Language Publ House, Moscow, 1956) with remarkable introduction & notes by Ch. Koschtajants, Professor of Physiology at Moscow State University.

Interesting Item: PN Witt offers *Die Wirkung von Substanzen auf den Netzbau der spirals biologischer Test* (Lange & Springer, Berlin W 35, 1956, 79pp, DM 16).

FEBRUARY, 1957

Preclinical & Clinical: S Akerfeldt notes oxidation of dimethyl-phenylenediamine by serum from mentally disturbed patients (*Science* 125/117/Jan 18/57). L Bartalanfy & Co propose acridine orange fluorescence technic for exfoliative cytology diagnosis of malignancy (*Science* 124/1024/Nov 23/56). DJ Bauer & PL Bradley note neurotropic viruses increase xanthine oxidase in brain, with grouping of viruses in order of increase (*Brit J Exp Path* 37/447/56). PO Bishop & WR Levick study single nerve fiber membrane potentials (*J Cell Comp Physiol* 48/1/56). EE Buckley & N Porges edit Berkeley symposium on *Venoms* (AAAS, Washington, DC, 57, 480pp, $9.5). C Else & E Scharrer edit 35th vol *Ergeb Anat Entwicklegesch* (Lange & Springer, Berlin, 56, 554pp, DM 248). H Frings & F Little note imobility of bees at sound frequencies between 550 & 800 cy/sec (*Science* 125/122/Jan 18/57). RG Grenell & LJ Mullins edit *Molecular Structure & Functional Activity of Nerve Cells* (Amer Inst Biol Sci, Washington, DC, 56, 205pp, $4.75). A Grollman edits *Clinical Physiology: The Functional Pathology of Disease* (McGraw-Hill, NY, 57, 800pp, $12). P Introzzi & P de Nicola well survey platelet physiolpathology with thrombelastography (*Sci Med Italica* 4/656/56). MA Jermyn gives mechanism of carbohydrase action (*Science* 125/12/Jan 4/57). HM Kalckar & Co analyze enzyme factors in congenital galactosemia (*Science* 125/105, 113, 116/Jan 18/57). AJ Kluyver & CB Van Neil describe *The Microbe's Contribution to Biology* (Harvard Univ Press, Cambridge, Mass, 56, 161pp, $4). CH Li & H Papkoff prepare growth hormone as protein with 26,000 mol weight from human pituitaries (*Science* 124/1293/Dec 28/56). AW Oughterson & S Warren note *Medical Effects of the Atomic Bomb in Japan* (McGraw-Hill NY, 57, 500pp, $8). EJ Orma & M Koskenoja review postural dizziness in the aged (*Geriatrics* 12/49/57). JT Park & JL Strominger propose mechanism of penicillin action by blocking biosynthesis of bacterial cell wall (*Science* 125/99/Jan 18/57). L Pauling & HA Itano edit *Molecular Structure & Biological Specificity* (Amer Inst Biol Sci, Washington DC, 223pp, $4.75). DM Pillsbury, WB Shelley & AM Kligman offer broad survey of *Dermatology* (WB Saunders, Philadelphia, 57, 1331pp, $20). H Popper & F Schaffner survey *The Liver: Structure & Function* (McGraw-Hill, NY, 57, 777pp, $20). MD Reynolds & Co with sensitive serum acid phosphatase test show significant rise in malignancy (*Cancer Res* 16/943/56). KM Richter reports effects of histamine & chlortrimeton on chick heart tissue culture (*J Cell Comp Physiol* 48/147/56). D Rudnick edits *Cellular Mechanisms in Differentiation & Growth* (Princeton Univ Press, 57, 248pp, $7.5). LH Rudy edits symposium on physiologic aspects of aging (*Geriatrics* 12/18-47/57). J Shields & E Slater investigate character of children of cousins (*Acta Gen Stat Med* 6/60/56) honoring 60th birthday Tage Kemp. IW Sizer notes chemical aspects of enzyme inhibition (*Science* 125/54/Jan 11/57). IH Slater & Co find phenaglycodol (2-p-chlorophenyl-3-methyl-2, 3-butanediol) is effective safe neurosedative (*Proc Soc Exp Biol Med* 93/528/56). WW Spink clarifies *The Nature of Brucellosis* (Univ. Minnesota Press, Minneapolis, 56, 464, $8). AA Wilcox & JM Luck show hepatoma formation is accompanied by reduced ethanolamine output (*Cancer Res* 16/926/56).

General: C Amory gives appreciation of Everette Lee DeGolyer 1886–1956 (*Sat Rev* Jan 26/57, pp 34–37). GW Beadle discourses on Uniqueness of man (*Science* 125/9/Jan 4/57). JS Bruno & Co make *A Study of Thinking* (Wiley, NY, 56, 330pp, $5.5). V Bush discusses professional collaboration (*Science* 125/49/Jan 11/57). SA Corson comments on SP Botkin (1832–1889) & the neurogenic theory of medicine (*Science* 125/75/Jan 11/57). T Dobzhansky gives *The Biological Basis of Human Freedom* (Columbia Univ Press, NY, 57, 135pp, $3). J Keats notes *The Crack in the Picture Window*, breaking mass produced suburban houses (Houghton Mifflin, Boston, 57, 215pp, $3). JW Krutch shows strength of *The Great Chain of Life* (Houghton Mifflin, Boston, 57, 277pp, $4). R Lyne wisely, wittily & wickedly describes *A Surfeit of Honey* (Harper's, NY, 57, 212pp, $3). JR Royce reviews current status of psychology (*Amer Sci* 45/57/57). May Sarton writes *The Fur Person* for cat-lovers (Rinehart, NY, 131pp, $2.75). H Selye describes *The Stress of Life* (McGraw-Hill, NY, 57, 320pp, $6). WL Thomas Jr edits *Man's Role in Changing the Face of the Earth* (Univ Chicago Press, 56, 1193pp, $13). RJ Williams describes *Biochemical Individuality* (Wiley, NY, 57, 214pp, $5.75).

March, 1957

General: AAAS Committee on social aspects of science makes preliminary report; any comments or suggestions? (*Science* 125: 143/Jan 25/57). EN Andrade gives early history of vacuum pumps (*Endeavor* 16/29/57). GE Bentley edits Dame Juliana Berner's *The Arte of Angling* (Middleton, London, 1577, 40pp) with introduction by CO Kienbusch, whose copy is unique (Princeton Univ Library, 56, 66pp). C Cherry reflects *On Human Communication* (Wiley, NY, 57, 333pp, $5.75). FS Crawford Jr shows experimental verification of clock-paradox of relativity; *i.e.*, to keep young, relatively, keep space travelling (*Nature* 179/35/Jan 5/57). HP Davis & HV Bracken give *Perspectives in Personality Theory* (Basic Books, NY, 57, 435pp, $6.5). E Fromm contributes to *The Art of Loving* (Harper, NY, 57, 185pp, $2.75). RW Gerard reports progress in drugs for the soul (*Science* 125/201/Feb 1/57). G Gleason offers *Horizons for Older People* (Macmillan, NY, 57, 205pp, $3). J Ladd analyses Navaho ethics in *The Structure of a Moral Code* (Harvard Univ Press, Cambridge, Mass, 57, 474pp, $8). G Lawton goes *Straight to the Heart* in telling about heart surgery (International Univ Press, NY, 57, 245pp, $5). EGP Lovejoy looks at *Women Doctors of the World* (Macmillan, NY, 57, 412pp, $6). J Nehru introduces *Nuclear Explosions & Their Effects* (Ministry of Information, Old Secretariat, Delhi 8, India, 57, 180pp, $1). I Progoff probes depth psychology in *The Death & Rebirth of Psychology* (Julian Press, NY, 57, 275pp, $4). GF White edits *The Future of Arid Lands* (AAAS, Washington, DC, 57, 464pp, $6.75). D Wolfle discusses science & public understanding (*Science* 125/179/Feb 1/57).

Clinical: JW Dundee & Co show anesthetic ad- & disad-vantages of "Fluoromar," trifluoro-ethyl-vinyl-ether (*Anesth* 18/66/57). FG Evans notes *Stress & Strain in Bones: Their Relation to Fractures & Osteogenesis* (CC Thomas, Springfield, Ill, 57, 245pp, $6). E Grafe offers *Ernahrungs- und Stoffwechsel-Krankheiten* (Springer, Berlin, 57, 1120pp, DM 100). HC Hagedorn analyses

management of diabetes mellitus (*Wein klin Wchnschr* 69/21/Jan 11/57). SH Kraines discusses *Mental Depressions & Their Treatment* (Macmillan, NY, 57, 592pp, $6). TT Tourlentes edits symposium on psychiatric aspects of aging (*Geriatrics* 12/101/57).

Preclinical: LK Dahl finds greater sodium intake in hypertensives than in normals (*Proc Soc Exp Biol Med* 94/25/57). CE Dalgleish notes time factors in protein biosynthesis (*Science* 125/271/Feb 15/57). EA DeFelice & Co indicate reserpine antagonizes effect of thyroid hormone on oxygen consumption (*Proc Soc Exp Biol Med* 94/171/57). JC Eccles discusses *The Physiology of Nerve Cells* (Johns Hopkins Press, Baltimore, 57, 298pp, $5.55). LE Ellinwood & Co find potassium is promptly released from x-irradiated mammalian heart (*Proc Soc Exp Biol Med* 94/129/57). BR Erickson & Co find animals in traumatic shock are poikilothermic with body temperatures approaching environments'—with 20° C optimum for survival (*ibid* p 1). CD Feuss & L Cragg find "Iniactin" (Oxanamide, Merrill; 2-ethyl-3-propylglycidamide) useful in quieting irritability in cerebral arteriosclerotics (*Dis New Sept* 18/29/57). A Goldin & Co show antileukemic action of reserpine (*Science* 125/156/Jan 25/57). CS Harrison says total body water equilibrium with injected deuterium oxide is reached only after 2 hrs in healthy or in thermally injured animals (*Proc Soc Exp Biol Med* 94/15/57). DD Jensen explores "learning" in paramecia (*Science* 125/191/Feb 1/57). WD McElroy & B Glass give *The Chemical Basis of Heredity* (Johns Hopkins Press, Baltimore, 57, 864pp, $12.5). F Sanger & LF Smith detail the structure of insulin (*Endeavor* 16/48/57). A Staub & Co note glucagon increases kidney output of electrolytes (*Proc Soc Exp Biol Med* 94/57/57). D Svorad shows functional activity of brain stem arousing system in animal hypnosis (*Science* 125/156/Jan 25/57). JB Thiersch finds o-diazo-acetyl-l-serine & 6-diazo-5-oxo-l-norleucine in low safe doses destroys rat litters in utero & causes abortion without injury to mothers (*Proc Soc Exp Biol Med* 94/27, 33/57). JA Thomas edits *Prolemes de Structures, d'Ultrastructures et de Fonctions Cellulaires* and *Croissance Cellulaire: Faceurs d'Activation et d'Inhibition* (Masson, Paris, 56, 358pp, 3,000 Fr & 57, 424pp, 4,000 Fr). TH Weller & Co isolate virus associated with cytomegalic disease (*Proc Soc Exp Biol Med* 94/4/57). RJ Wulf & RM Featherstone show how Van der Waals constants correlate with anesthetic potency, explaining anesthetic action of xenon (*Anesth* 18/97/57). JM Yoffey & FC Courtice discuss *Lymphatics, Lymph & Lymphoid Tissue* (Harvard Univ Press, Cambridge, Mass, 57, 510pp, $10).

C. D. Leake

ERRATA

In volume 15, number 2, two errors occur in the article by Arild Hansen and Mary Haggard. On page 271 fifth line from the bottom should read "1889." Page 280, 23d line down should read 80 or 90 instead of 80 to 90.

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MEDICINE, MEN, AND THE VITAL REVOLUTION[a]

ROBERT C. COOK[b]

The whole world is aware of the destructive potential of nuclear weapons. Somehow that suicidal potential must be transmuted into a realistic atoms-for-peace plan if modern man is to have any hopeful future. If that is accomplished, and it *must* be in the years just ahead, the atomic age could put at man's disposal vast new sources of power exceeding many times the reserves of the fossil fuels—coal and oil—which turn the wheels of industry today. Once this destructive potential has been successfully harnessed for peaceful purposes, our creative energies must be centered on averting the slow working, insidious dangers posed by the "vital revolution."

It has been said that the "population bomb," the chain reaction increase in numbers of people, can in the long run be as destructive to the human race as the hydrogen bomb. Fortunately its fuse is longer, for biological chain reactions have a different timing. That longer fuse can enable mankind to save himself, if he acts intelligently without wasting the precious minutes as they tick into hours and years.

The man of medicine, whether he likes it or not, sits squarely in the middle of this modern dilemma. Will he rise to the challenge which the vital revolution has thrown at his feet, or will he, already overburdened as he is with specialization, choose to let this great opportunity pass by default? If he accepts the challenge then he must acquaint himself with the basic facts of demography and genetics—two relatively new sciences which impinge increasingly on the medical man's area of interest.

During the first half of the twentieth century, medicine played a major role in changing the life patterns of human beings. In many countries today, and potentially in others all over the world, death in childhood and youth has been drastically reduced. Where a quarter of the children usually died before their first birthday a century ago—in bad years often a half—today in the United States more than nine-tenths of the babies reach voting age.

In most of the industrialized countries today, birth has become increasingly a matter of choice rather than the too often unwanted by-product of primal urges and ignorance. This, too, could spread to all countries. The dramatic change in patterns of birth and death ushered in the "vital revolution" through which mankind is now passing.

What are the dangers of the "vital revolution"? The most urgent is the phenomenal increase in the rate at which human beings are multiplying. This is a direct result of the discovery of ever more efficient methods of death deferment during the past century.

People are obsessed with the idea that population increases occur only through an increase in births, and that birth rates throughout the world are rising. Actually, in the industrial countries, birth rates have been tending downward for a century. The current "baby boom" in the United States is an exception.

In the so-called "underdeveloped countries," birth rates, have always been and still are near a physiological maximum. The dramatic increase in numbers of people, and the speed-up in this increase year after year, is due to *declines in death rates*. Modern public health methods coupled with modern technology have cut the death rate by a third to a half in a single year in some countries where birth rates have been, and remain, high. The result is a startling increase in the rate of population growth.

Ceylon is a classic example. There a malaria control campaign dropped the death rate from over 20 per thousand of the population to about 14 in one year, 1945–46. It has since continued to edge downward. The birth rate has stood near 40 for many years. It has not declined; if anything it has actually increased slightly since 1950. As a result, population growth skyrocketed to over three per cent a year in this small, already overcrowded island country which produces only about half the food necessary to sustain 300 people per square mile, in contrast to 50 people per square mile in the United States.

The present rate of increase in Ceylon's population portends ultimate disaster, for the population will double at the present rate of increase in less than 20 years. The only alternatives to an eventual Malthusian tragedy are either a reduction in birth rates or mass emigration. The latter solution as a permanent "out" is purely imaginary. The speed-up of population growth is wide-

spread throughout the world and the siphoning off of multimillions of people each year to shrinking unoccupied areas is neither feasible nor a permanent solution of the problem. From India alone, five million persons per year would have to be moved out to keep the population in balance. That is at the rate of 14,000 per day from one Asian country alone.

The other alternative is to develop effective means of fertility control which can be used to balance the increasingly effective death deferment techniques developed by modern science. Without such a balance present trends are heading many hundreds of millions of people towards mass hunger and ultimate starvation.

It is obviously not an enlightened approach to ignore this tremendous problem and to hope that some miracle of loaves and fishes will make it unnecessary for humankind to exercise reason and foresight. Default rather than enlightened action merely invites Malthusian checks. No humane person wants to see the crisis resolved by a rise in death rates in his country or anywhere on earth.

This population problem, generated by the increase in medical knowledge and its widespread application, is definitely a medical problem, even though its solution lies mostly outside the area of medical practice. The understanding and the cooperation of physicians is needed if humane and effective solutions are to be developed in time to avoid disaster. Today the daily current net increase of births over deaths in the world amounts to some 85 to 100 thousand people. That is the equivalent to the population of Alexandria, Virginia. Obviously, a compound rate of increase of this magnitude cannot go on indefinitely. Even now hunger is becoming more widespread in Asia, though outright famines are still being prevented. But the trend is ominous. Physicians, pledged to prevent suffering and death, dare not ignore this problem.

Another aspect of the vital revolution which concerns the medical profession is the question of maintaining the hereditary fitness of the human race. Evolution has taken place in living organisms, including man, by the cruel and stringent method of death selection. The variation upon which selection acts is produced by the kaleidoscopic recombination of genes in the process of sexual reproduction, and by the constant production of new mutations at a slow rate. It may seem paradoxical that only very

rarely are mutations advantageous to the organism. So that a continuous sifting out of the deleterious mutations is perhaps as important a part of the selective process as the bringing together of advantageous combinations of genes by selective reproduction. X-rays and nuclear fall-out can greatly speed up the mutation process, but this cannot be explored here.

This would mean that there would be a slow increase in the frequency of death and disability due to genetic causes. The distinguished geneticist, Dr. H. J. Muller, has recently estimated that about 20 per cent of births must be selectively eliminated either by death or by non-reproduction to avoid an accumulation of morbid genes in the population. Since less than 10 per cent of the population fails to reach reproductive age and since many deaths are non-selective, there would appear to be an inevitable accumulation of morbid heredity even if Dr. Muller's estimate errs on the pessimistic side. Over many generations this could pose serious medical problems and result in the eventual biological disintegration of the human race. Fortunately, this danger is not imminent nor need it involve the crude and cruel expedient of "death selection." For birth selection, if effectively and humanely applied, can be even more effective. It appears that among all members of the human species, the desire for perfect children is so strong that few women can face with equanimity the prospect of bearing malformed or afflicted children. As knowledge of human genetics becomes more adequate, and as the facts of genetics become more widely known, one may hope that voluntary abstinence from parenthood will become the rule in those instances where there is adequate evidence that serious hereditary defects will be transmitted to future generations.

At the present time, we do not know enough about human genetics to formulate a comprehensive program for the long-term conservation of the genetic heritage of our race. Enough is known, however, so that in some family lines, where serious defects have been transmitted for generations, a voluntary stoppage of such continuing tragedies has taken place. No easy solutions are likely to be found. Obviously, this is an area where the difficulties are great, and where knowledge will have to be applied with the utmost delicacy. Nor can the implications of what is now known and what is being discovered in this field be safely ignored.

Fortunately, this problem of maintaining the genetic fitness of the human germ plasm is a long term one which does not demand drastic action this year or next. Even though the build-up of morbid genes is mercifully slow, medical men do have a responsibility to learn the basic facts of genetics so they will be in a position to give—or to obtain from specialists—competent counsel to their patients in those individual cases where existing knowledge permits definite predictions to be made. Pedigree analysis makes it possible to state in some cases that a particular individual will transmit serious genetic defects to his descendants. Again, individuals in the "tainted" line may run no risk of transmitting the defect. Where genetics can give a definite warning or a reassuring "all clear" the patient has a right to this knowledge.

This does not mean that the already overburdened general practitioner must become a genetic specialist. The job of the physician is to be adequately grounded in genetic principles so that he can recognize when a genetic specialist is needed. The interpretation of the available data is the province of the expert, just as the localization of a brain tumor requires the skill of a neurologist. Genetic specialists are now to be found in some six institutes of human genetic research in the United States where consultations and analyses of pedigrees are now available.

With the emergence of human genetics, medicine will become not only the conservator of the patient's health but increasingly the conservator of the health of future generations: public health with a new dimension added—*time*. With an increasing understanding of genetics, the practice of medicine will gain new insight and interest.

This writer is passionately opposed to the proposition that such matters can be handled by commission, committees, or legislatures. The need is for widespread knowledge and for adequate counseling. The responsibility to disseminate and interpret this knowledge should be assumed in considerable part by the medical profession.

Each patient who appears in a physician's waiting room is, after all, an unique collection of human genes. So diverse is the human germ plasm that no two people are exactly identical. The responses which patients make to various foods and drugs, to the crises of living, and to the daily pressures of keeping up with the

Joneses and ahead of the collectors of bills and taxes, are in part conditioned by their genetic makeup. A knowledge of genetics not only will open up new vistas of understanding of the individual patient, it will add a new challenge and fascination to the physician's career.

REFERENCES AND NOTES

[a] Reprinted by permission from The Courier of George Washington University, Wash. D.C.

[b] Lecturer on Genetics, Director, Population Refrence Bureau, Inc., 1507 M St. N.W., Washington, D.C.

ELECTRON MICROSCOPE STUDY OF THE EMBRYONIC NEURAL TUBE AND NOTOCHORD[a]

DONALD DUNCAN[b]

Thanks to the pioneer work of Pease and Baker in 1948 and a subsequent succession of improvements in ultrathin sectioning combined with equally important advances in fixation, anyone with material prepared by present-day methods and an electron microscope can gaze at a world unknown a decade ago. If one's sections are from material not previously studied by this method, heretofore unseen structures are apt to be revealed. In addition, some structural details that are questionable under the light microscope can be expected to stand out with undisputable clarity.

With these points in mind, chicken embryos were prepared for study with the electron microscope. Besides any new features that might thus be found, it was hoped that evidence would be obtained for complete and definite boundaries between cells of the neural tube as maintained by Sauer (1935). Sauer's description is at variance with an earlier and widely accepted opinion advanced by Hardesty (1904) to the effect that neural tube cells are syncytial.

Material and Methods

Chicken eggs were incubated 24–72 hours and then fixed either in Palade's (1952) veronal acetate buffered osmium tetroxide or in Dalton's (1935) osmic dichromate mixture. The fixative was applied directly to the exposed embryos in some instances, in others the embryo was removed from the egg and immediately immersed in the fixative. Within a few minutes after initiation of fixation, each embryo was transversely sliced into pieces one-half to three millimeters in length. Total fixation time varied between thirty minutes and four hours. Subsequently, the material was washed briefly first in Ringer's salt solution and then in distilled water. Dehydration was started with 70 per cent ethyl alcohol and continued through absolute, three changes, and from there to a mixture of 15 per cent methyl methacrylate in n-butyl methacrylate without catalyst. After three changes of the uncatalyzed plastic, the material was placed in the same mixture of methacrylate, with 1 per cent Luperco CDB[c] added as the catalyst. After one or more changes of catalyzed plastic the material was transferred to No. 00 gelatine capsules. The capsules

were placed in a 46° F. oven until the plastic was sufficiently hard for cutting. The material was sectioned on a Porter-Blum microtome and picked up on copper grids. The sections were supported on the grids either by thin collodion film or by coating the unsupported section with carbon as recommended by Low (1956). The sections were examined and photographed in an RCA EML 1B microscope.[d]

Results

As illustrated by figure 1, the notochord in 36–72 hour chick embryos is surrounded by a halo of extracellular material that is not found adjacent to the neural tube in the earlier stages examined; *i.e.*, 36 hours or younger. In somewhat later stages, similar material partly surrounds the neural tube apparently by extension from the notochord. No material of this or any other kind was seen elsewhere, and all parts of the embryo were examined.

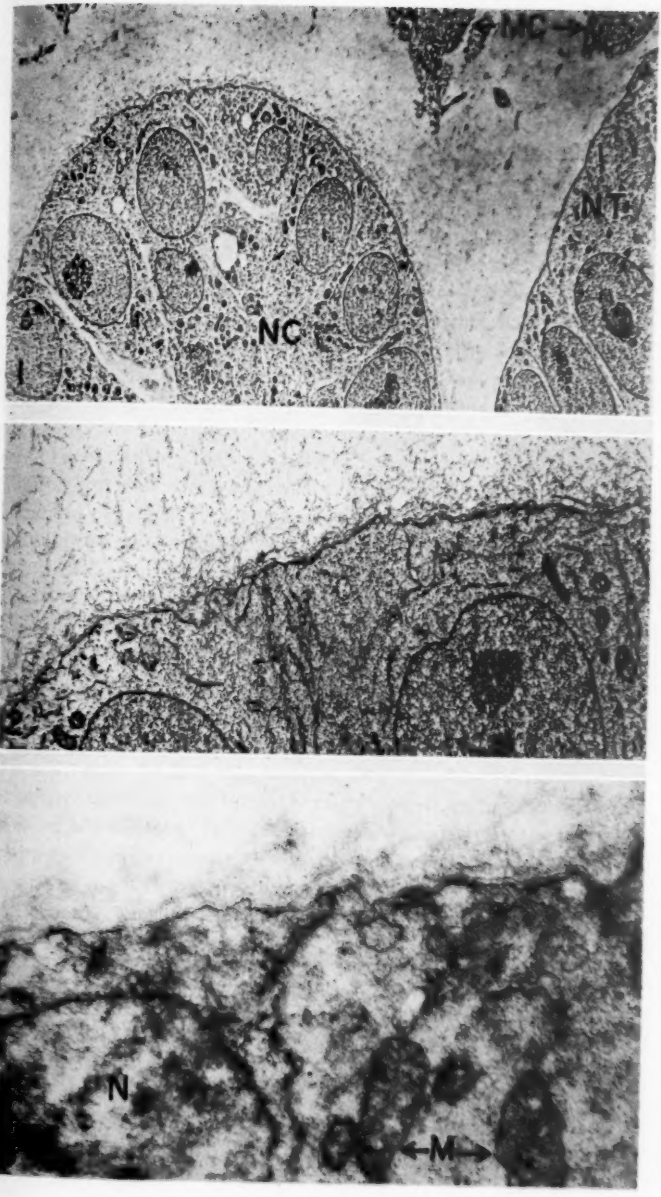
The perinotochordal material in many embryos appears to be composed of very fine fibrils without discernible internal structure or signs of periodic changes in density or diameter (Figs. 2 and 3). In other preparations, the fibrillary nature of this material is not so apparent. In these the appearance is that of a fine irregular network similar to that obtained by coagulation of protein solutions in formalin and many other fixatives. Differences in the appearance of the extracellular material have not been satisfactorily accounted for; although, it is believed that slight differences in fixation and subsequent washing may be responsible. Structurally, the material is not collagen although Mallory's connective tissue stain shows a brilliant blue line in the same position. In ten somite and still younger embryos, extracellular material of this type has not been demonstrated, but fixation of these stages has never been completely satisfactory.

PLATE 1

FIG. 1. A low power view of portions of the notochord, NC; neural tube, NT; and bits of three mesenchymal cells, MC. Note a halo of finely divided extracellular material around the notochord and its absence elsewhere. 36 hour chick. X 2,400.

FIG. 2. A portion of the notochord from a chick 36 hours old. In this view the extracellular material is in the form of very fine irregularly arranged fibrils. X 10,000.

FIG. 3. Margin of the notochord of a 48 hour chick showing a limiting membrane consisting of an inner dense line, an outer dense line, and a clearer interval between them. The extracellular material at the left side of the picture is quite suggestive of origin by successive delaminations of the outer limiting membrane. A nucleus is indicated by N, mitochondria by M. X 27,800.


NC
NT
NC
N
M

The limiting membrane of the notochord (Figs. 2 and 3), as well as the external limiting membrane of the neural tube, is a triple affair consisting of two dense lines separated by an interval of much lower density. In both structures the inner dense line is more pronounced than the outer one. There are no discernible differences between notochord and neural tube with respect to these limiting membranes and casual observation indicates that all epithelial structures of these embryos are similarly bounded. This type of membrane has been seen in the youngest stage examined; namely, the two somite stage.

The question of boundaries between adjacent cells in the neural tube was more difficult than anticipated. Many otherwise satisfactory preparations failed to demonstrate them convincingly. However, examination of much material indicates that fixation and sectioning must be close to ideal and the exact plane of section must be fortunate to demonstrate the intercellular boundaries of neural tube cells. Figures 4, 5, and 6 are submitted as evidence that such boundaries can be found. In figure 4 one cell shows complete boundaries except for one or two breaks evidently of an artifactual nature. The same holds for one or more cells shown in figures 5 and 6. One of the cells in figure 6 is a good example of the extreme attenuation required if all cells at this stage of development are to maintain contact with the neural canal.

Cell boundaries in the form of terminal bars are a prominent feature of the juxtaluminal zone of the neural tube (Fig. 7). They do not differ from earlier descriptions of these structures by many authors using different material. Most of the luminal surface is undifferentiated except for fairly numerous minor projections and indentations of the surface. However, in one specimen a few cilia-like projections were seen in the region of the

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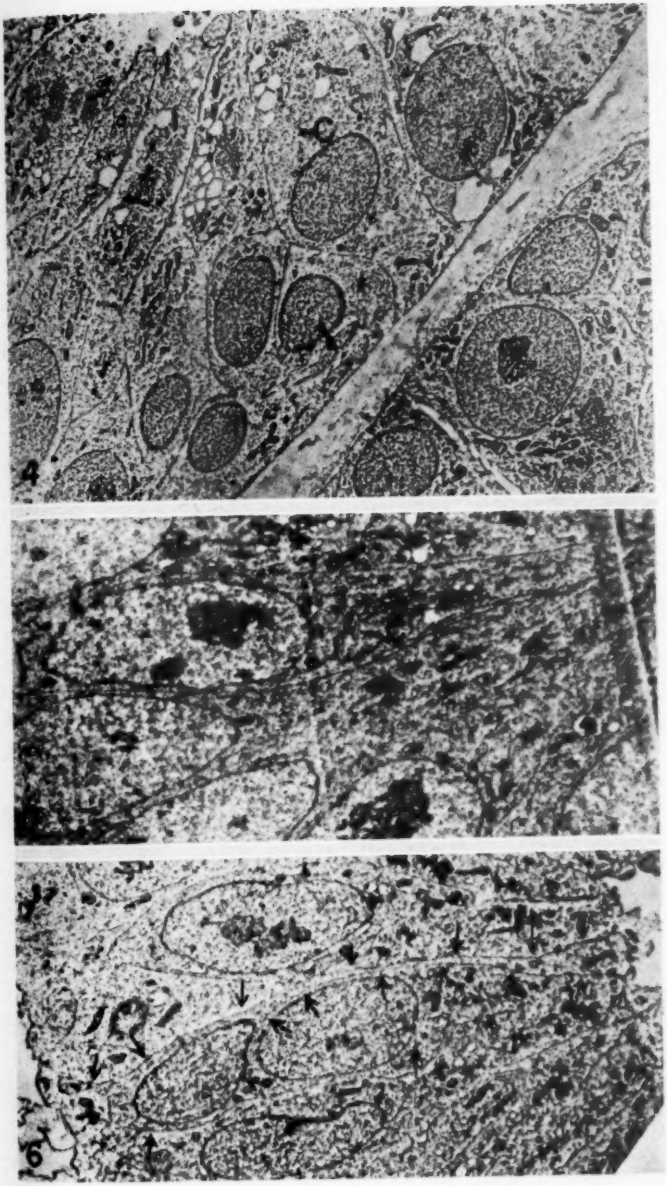
PLATE 2

FIG. 4. A low power view of adjacent portions of the neural tube and notochord. Boundaries between neural tube cells are quite apparent, particularly those of cell labeled C. 48 hour chick. X 3,800.

FIG. 5. This is a thicker section of the floor plate of the 48 hour neural tube. Note how cell boundaries are distinct for cell labeled C but are indefinable for adjacent cells. X 6,500.

FIG. 6. From the floor plate of a 48 hour embryo. The boundaries of a cell extending from lumen to external limiting membrane are indicated by arrows. X 4,400.



junction between alar and roof plates (Fig. 8). Under lower powers, the plasma membrane of the surface appears as a single dense line but a few pictures taken at higher power, and of the best fixed material indicates that this membrane can be resolved into two dense lines and an intermediate less dense material. This characteristic of free surfaces has been clearly depicted by Rhodin and Dalhamn (1956) in rat trachea; also, by Sheldon (1956) in mouse cornea.

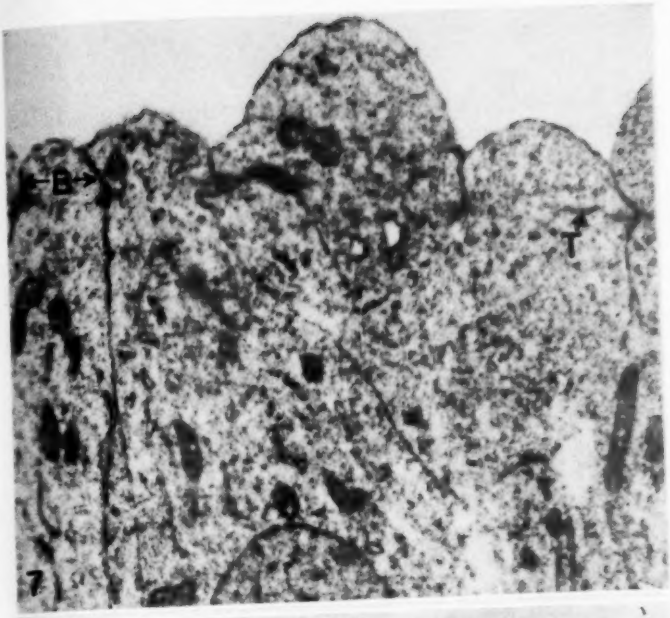
Many views of the juxtaluminal cytoplasm show a hazy density extending from one terminal bar to another (Fig. 7). The significance of this is unknown but may represent the terminal web described by Sauer and by de Renyi (1935).

All of the usual internal features of cells when viewed in the electron microscope were identified in this study. The mitochondria show no special features and the differences between those of the neural tube and notochord are what might be expected. The mitochondria of notochord are more irregular in shape and contain more small dense bodies than there are present in neural tube mitochondria. These bodies are such an inconspicuous feature of the neural tube mitochondria that the initial impression was that they are absent. The Golgi apparatus is not conspicuous but can be found in the form of very fine parallel membranes unassociated with vacuoles or conspicuous granules (Fig. 9). The endoplasmic reticulum (in both organs) is in the form of minute tubes encrusted with the small granules of Palade (1955) (Fig. 10). Similar granules are found throughout the cytoplasm. Nuclear membranes are characteristically double and possibly perforated. One clear example of continuity of the outer nuclear line with the limiting membrane of an endoplasmic tubule as first described by Watson (1955) is shown in figure 10. Despite considerable study of mitotic figures none of the finer details of chromosome structure such as described by Moses (1956) and by Fawcett (1956) have been found.

PLATE 3

FIG. 7. Luminal surface of a 48 hour neural tube to show terminal bars, B, and a hazy density, T, of the cytoplasm that may represent the terminal web of Sauer. X 25,000.

FIG. 8. A portion of the luminal surface of a 72 hour neural tube bearing a projection thought to be the beginnings of a cilium. X 33,000.



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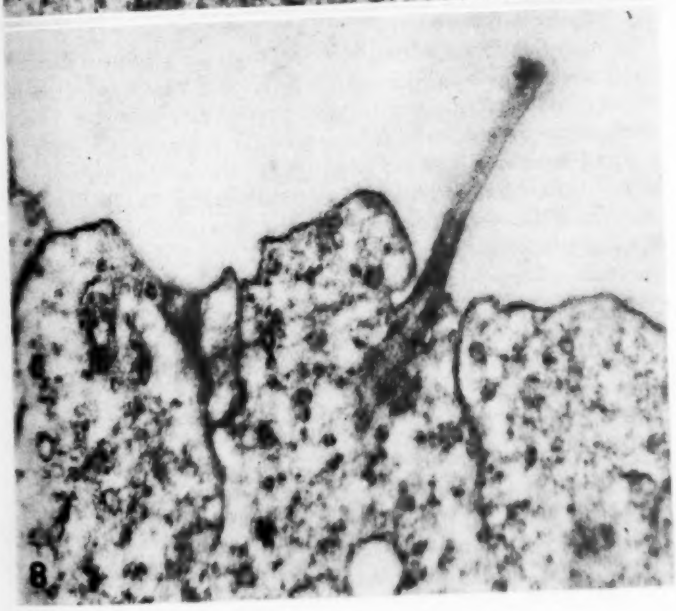

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Discussion

It is believed this study establishes three previously undescribed or disputed structural details. First of all, the observations support the epithelial versus the syncytial nature of the neural tube. While it is a fact that many preparations and many pictures fail to establish discernible cell boundaries between all cells, it is equally true that favorable preparations exhibit these boundaries with great clarity. It seems reasonable, therefore, to consider apparent absences of cell boundaries as artifacts of preparation. The alternative is to hold that some cells lack boundaries and some have them. Also, that some cells will have well established limits in some places and lack them in others. While such an opinion is tenable until refinements of technique permit unfailing demonstrations of boundaries in every instance, it is considered highly improbable.

The double, or rather triple, nature of the external limiting membrane of epithelial structures was probably first described by Ottoson, *et al.* (1953), and subsequently by many others. Robertson (1956) gives an excellent description of a similar structure for sarcolemma.

In material derived from older animals such as used for previous descriptions of this triple line, one might question its source; *i.e.*, derived from parenchyma or from stroma. In the young embryos the same arrangement is present as the external limiting membrane of the neural tube and without any possibility of contribution from extraneural sources. While the outer dense line has been designated as the basement membrane by Ottoson and others, it should be kept in mind that it is not the structure referred to in descriptions based on the light microscope.

Since the notochord is the primitive skeleton of all chordates, it might reasonably be expected to initiate the production of

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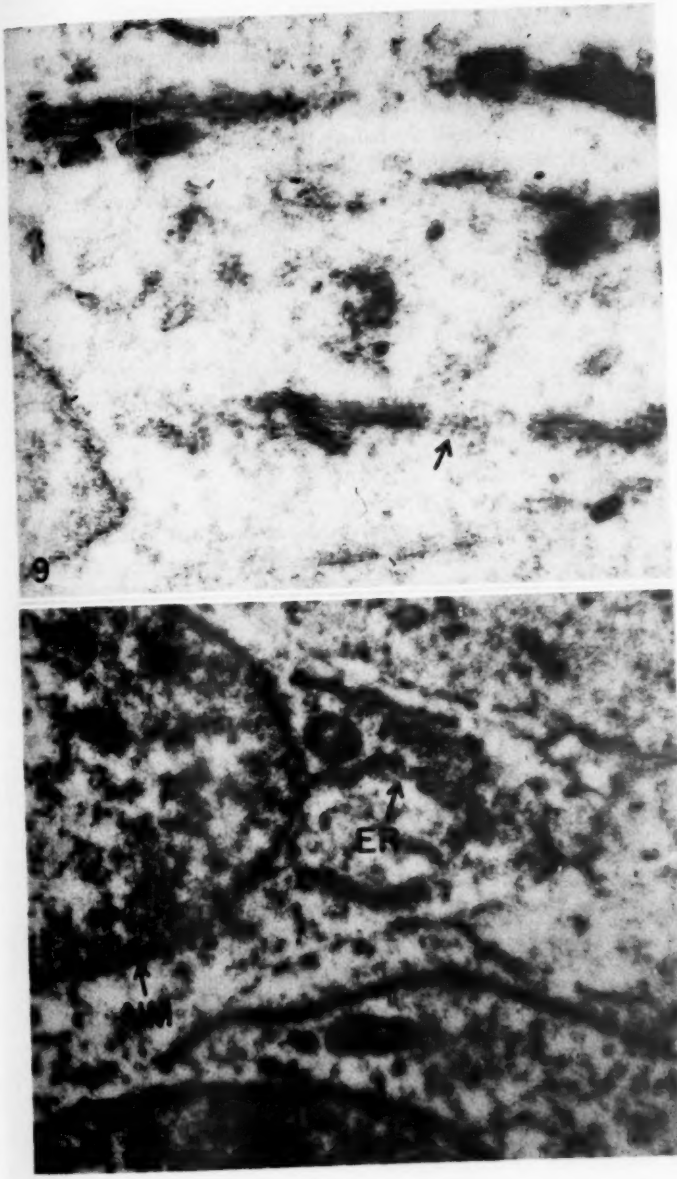
PLATE 4

FIG. 9. A portion of a neural tube cell displaying fine parallel membranes and considered to represent the Golgi apparatus. As indicated by the arrow these membranes may be tubular. X 33,000.

FIG. 10. A portion of the notochord at 36 hours to illustrate continuity between outer nuclear membrane, NM, and endoplasmic reticulum, ER. X 38,000.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ER
PM

extracellular material. In fact, it has been experimentally demonstrated by Avery and Holtzer (1956) that chick notochords of ages described here are capable of inducing cartilage formation by cells from the somites before they have acquired this power independently. The electron microscope appearance of the notochord may be visible evidence of such an inductive substance or merely as associated phenomenon. It might be that contact with this material causes other cells to elaborate similar material at their surfaces. The pictures give no real proof of how this extracellular material is formed but they do suggest some type of shedding process from the surface, possibly a repeated peeling off of the external dense line of the limiting membrane. To what extent this process is akin to collagen formation will require much further study.

Summary

Chicken embryos approximately 24–72 hours old were prepared for study with the electron microscope. During these stages, the external limiting membrane of the neural tube and the limiting membrane of the notochord appear as two electron dense lines separated by an interval of lesser density. The notochord appears to be shedding a fibrillary extracellular material that is seen nowhere else in the embryo. The luminal surface of the neural tube is characterized by prominent terminal bars, by small projections and indentations of the surface membrane, and an occasional developing cilium. This surface membrane is an electron dense line that occasionally appears as two finer lines separated by a clearer interval. The study indicates an epithelial rather than a syncytial structure of the cells of the neural tube.

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[a] Supported by USPHS Grant No. B-690(C2).

[b] Department of Anatomy, The University of Texas Medical Branch, Galveston. Accepted for publication May 16, 1957.

[c] Manufactured by the Lucidol Division, Wallace and Tiernan, Buffalo 5, N. Y.

[d] Purchase of this instrument was made possible by a generous donation from the Brown-Lupton Foundation.

A CASE OF HUMAN MYIASIS IN TEXAS CAUSED BY THE PRIMARY SCREWWORM, *CALLITROGA HOMINIVORAX* (COQUEREL)

GEORGE W. HUNTER III[a] AND CARLOS M. BERROCAL[b]

Myiasis is a well recognized condition that is produced by dipterous fly larvae when they invade the organs or tissues of animals or man. *Callitroga hominivorax* (Conquerel) (= *Callitroga* [*Cochliomyia*])*americana* (Cushing and Patton), the primary screwworm, is one of the more important members of the family of blowflies, the Calliphoridae. The members of this group are obligate parasites and this species is probably the most serious myiasis-producing screwworm of the Western Hemisphere (James, 1947). Knipling and Rainwater (1937) report that about 90 per cent of 901 cases of wound myiasis occurring in animals and man were caused by *C. hominivorax*. Only when there is a severe outbreak among livestock is there likelihood of encountering many cases of human myiasis. Such a situation was found in 1935 by Dove (1937) when he reported 55 cases of screwworm infestation in man in the South and estimated that there were probably two times as many undetected cases. Emmett and Siverly (1952) reviewed 39 cases which included those of Yount and Sadler (1907) from Arizona and reported 13 per cent mortality in this group. Taylor (1938, 1950) gave data on 8 cases from Florida and Turnley (1954) discussed screwworm infestations in man. Micks and Calma (1952) added 3 more records of myiasis of the nose of man from Texas. We add one more cases of human myiasis to the Texas records.

Case Report

M. F., a 69-year-old Caucasian female, was first admitted to the San Antonio State Mental Hospital in 1949. At the time of admission she had psychoses and arteriosclerosis. On November 9, 1954, the patient was transferred to Clinic No. 3 with a large, sloughing infected hematoma on the left side of the frontal region of the scalp. The wound was filled with maggots which

were manually removed daily for five days. The wound then became clear and free of infection, granulating nicely. Diagnosis: Infected hematoma of scalp. Condition improved. Patient transferred.

These fly larvae were tentatively identified as *Callitroga hominivorax* (Coquerel) (=*Callitroga* [*Cochliomyia*]) *americana* (Cushing and Patton).[e]

Discussion

Unfortunately the exact number of maggots recovered is not known. However, 68 were recovered on November 9th (the day the patient was transferred to the clinic) and 15 reported two days later; this accounts for 83. It is known that some were recovered *prior* to the transfer of the patient to the clinic and the clinical record states that maggots were found for five days, *i.e.*, November 9–14. The eggs of *C. hominivorax* are typically deposited in batches on injured or even unbroken skin; they hatch in less than a day and invade the skin where they produce festering, frequently deep, disfiguring wounds. One fly may lay up to 300 eggs within five minutes. In view of this it appears probable that only a single fly was involved. It is known that the larvae require up to three weeks (usually 4–10 days to complete their development) depending upon the conditions encountered. When fully grown, they leave the host and pupate in the soil. Since the mature larvae were first observed on November 9, one may speculate that the infection probably occurred 4–10 days earlier.

Summary

A case of human myiasis caused by *Callitroga hominivorax* (Coquerel) is reported from a woman in San Antonio, Texas.

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[a] Col., U.S.A. (Ret.). Department of Microbiology, College of Medicine and Department of Biological Sciences, University of Florida, Gainesville.

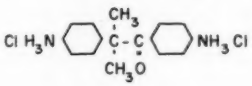
[b] Agricultural Experiment Station, University of Puerto Rico. Formerly Laboratory Consultant, San Antonio State Mental Hospital and Laboratory Director, San Antonio State Tuberculosis Hospital. Accepted for publication April 1, 1957.

[c] This was confirmed through the courtesy of Dr. W. W. Wirth of the Plant Industry Section of the Entomology Research Branch of the Agricultural Research Service of the USDA.

BRIEF RECORDING: THE SHORT TERM EFFECT OF AMPHENONE IN A PATIENT WITH CONGENITAL ADRENAL HYPERPLASIA[a]

JOHN E. JOHNSON, JR., A. R. REMMERS, GEORGE W. CLAYTON, AND RAYMOND GREGORY

Amphenone is a substituted desoxybenzoin compound (Fig. I) with progestational, antithyroid, and central nervous system depressant properties (5, 7). In addition, it has the unique property



AMPHENONE

FIG. I.

of interfering with adrenal cortical steroidogenesis (6). Amphenone has been shown to interfere with the 11B-, 17a-, and 21-hydroxydation mechanisms, as well as oxidation of the △5-3B-hydroxyl group to the △4-3-ketone, all of which occur in the course of normal steroid hormone synthesis in the adrenal cortex (12). The administration of amphenone in man causes a sharp fall in the plasma level and urinary excretion of 17-hydroxycorticoids (5). Furthermore in patients with clinical hyperadrenocortical states (Cushing's syndrome), during the periods of amphenone suppression of 17-hydroxycorticoids, glycosuria is abolished, insulin requirement is reduced, elevated blood pressure falls to normal, and in one patient a state resembling the cortisone-withdrawal syndrome was produced (6). However, the excretion of 17-ketosteroids is less affected by amphenone (5, 9).

The syndrome of congenital adrenal hyperplasia is characterized by the urinary excretion of large quantities of 17-ketosteroids and of pregnanetriol (1). It was therefore of particular interest to study the effect of amphenone on steroid production in a patient with this condition.

Case Report

The subject of this study was a 31 year old woman who was first seen in this clinic at the age of nine months by the late Dr. James E. Thompson because of a genital anomaly consisting of clitoridal enlargement with the urethra opening at its base, and neither testes nor vagina apparent. At the age of nine at another hospital, the clitoris was amputated, female internal genitalia were identified at laparotomy, and the vagina was opened. Descriptions of this patient and two similarly afflicted cousins were reported by O'Farrell in 1935 (10). She was not seen again until 1954 when she came to learn if anything could be done about her virilized state. Her body contour was that of a man except for moderate breast development. Hairline was receding, and a bald spot was present on the vortex. She shaved daily, and pubic hair was male in type. Her voice was husky. She menstruated monthly with scant flow. She had been otherwise in good health, and physical examination was not otherwise abnormal. Urinary excretion of 17-ketosteroids was 46 and 62 mg. per 24 hours on two consecutive days (normal 8-16 mg. per 24 hours), which increased to 105 and 75 mg. per 24 hours during two days administration of ACTH. After daily administration of cortisone, urinary 17-ketosteroid excretion fell to levels of about 10 mg. per 24 hours. During the next two years on this regime, head hair growth increased, the bald spot disappeared, and growth of body and facial hair decreased. Body contour became less masculine.

In 1956 she entered the hospital to receive amphenone.[b] Urine was collected as 24 hour specimens and analyzed for 17-ketosteroids (3), 17-hydroxycorticoids (11) and pregnanetriol (2).

Results

Experimental conditions and results are plotted in Figure II. At the time of admission, 17-ketosteroid excretion was controlled by the weekly administration of 200 mg. of hydrocortisone acetate, which was continued throughout the study. The first two days represent control steroidal excretion under hydrocortisone suppression. On the third day, the daily administration of ACTH gel 80 mg. per day was begun in order to obtain maximal adrenal steroidogenic activity. This occurred after about eight days, as shown by peak values for all steroids measured. On the 13th and 14th days, five grams of amphenone was given intravenously over a five hour period on each day. Concomitantly the excretion of pregnanetriol and 17-hydroxycorticoids decreased sharply, but 17-ketosteroids did not change significantly. During the succeeding two days that ACTH was continued, pregnanetriol excretion increased, but did not reach previous peak levels before the study was discontinued. No change occurred in serum electrolytes, liver function tests or recumbent blood pressure, but

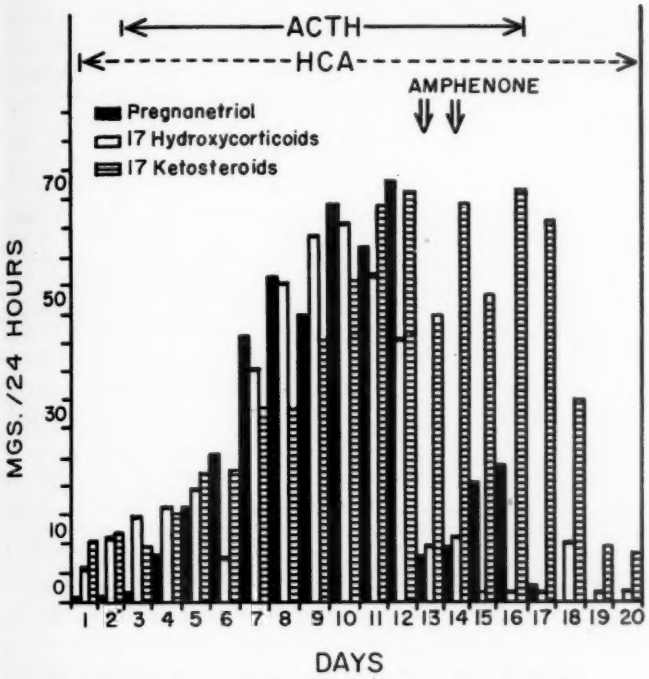


FIG. II. Effect of amphenone on urinary excretion of steroids.

a transient postural hypotension was noted on the second day of amphenone administration.

Discussion

The defect in steroidogenesis in patients with congenital adrenal hyperplasia consists in impaired formation of hydrocortisone-like steroids which normally regulate production of ACTH. According to Bongiovanni (1), the elevated level of pregnanetriol in this patient following ACTH would reflect accumulation of abnormal metabolites because of an enzyme block at 17-hydroxyprogesterone and 21-desoxyhydrocortisone, as shown in figure III. Since the known effects of amphenone include not only interference with hydroxylations at the 11B-, 17a,- and 21 positions, but also interference with oxidation of the △5-3B-hydroxyl

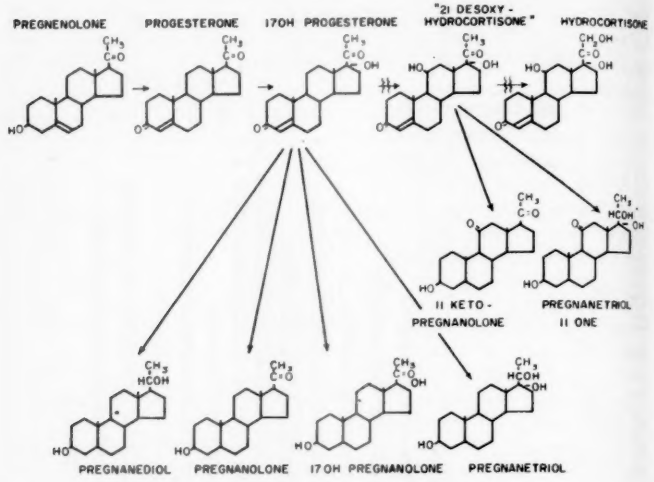


FIG. III. Scheme of adrenal steroid hormone synthesis, adapted from Bongiovanni (1).

group to the △4-3-keytone (12), suppression of pregnanetriol excretion would be expected to follow its administration. The rapid return of pregnanetriol excretion after discontinuing the drug indicates the reversibility of amphenone suppression. We (8) and others (5, 6) have observed reversibility of 17-hydroxycorticoid suppression after much longer amphenone administration.

The excretion of 17-ketosteroids is usually not much affected during amphenone administration (5). Hertz and his coworkers (5) observed an increase in 17-ketosteroid excretion in one of 24 patients who received amphenone, and we (8) have seen a striking increase in the 17-ketosteroids of a patient with adrenal carcinoma while receiving amphenone. Lanthier and his associates (9) found it necessary to remove an interfering metabolite of amphenone, after which they found no suppression of 17-ketosteroids by amphenone in normal persons, but a marked decrease in 17-ketosteroid excretion of two patients with adrenal carcinoma. There was a decrease in dehydroepiandrosterone and 11-oxygenated 17-ketosteroids, marked fall in the dioxane hydrolyzable fraction, and a slight fall in the ketodase fraction.

Similarly, in a patient with adrenal carcinoma Gallagher and coworkers (4) found a 95 per cent suppression of dehydroisoandrosterone, and 50–60 per cent decrease in etiocholanolone and androsterone during amphenone therapy.

Summary and Conclusions

The reversible suppression of pregnanetriol excretion by amphenone in a patient with congenital adrenal hyperplasia is reported for the first time. Excretion of 17-hydroxycorticoids was also suppressed, but 17-ketosteroids were not affected. Amphenone suppression of pregnanetriol could be predicted by its known effects on steroidal transformations within the adrenal cortex.

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[b] Amphenone was generously supplied by Dr. Roy Hertz.

ULTRA-VIOLET FLYING SPOT TIME LAPSE MOTION PICTURE OBSERVATIONS OF LIVING CELLS[a]

P. O'B. MONTGOMERY, W. A. BONNER,[b] F. ROBERTS[c]

In other communications the authors have described the technic of ultra-violet flying spot television microscopy (1, 2), which, it is claimed, has the two advantages: 1) ease of focusing in ultra-violet; 2) greatly reduced irradiation of the specimen. The purpose of this paper will be to add to the previously described technics a description of the technic of time lapse motion picture photography utilizing the above advantages of the ultra-violet flying spot television microscope, and to report the results of preliminary observations made on living cells.

Methods

The ultra-violet flying spot microscope (see Fig. I) consists of: A, an ultra-violet emitting scanner tube; B, an ultra-violet filter; C, a front aluminized

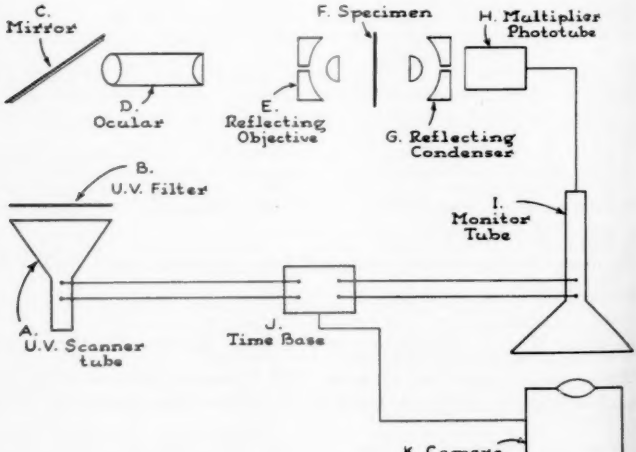

FIG. I. Block diagram of ultra-violet flying spot television microscope.

mirror; D, E, F and G, a reflecting objective microscope where D is the ocular, E and G the reflecting objective and condenser (of numerical aperature .72), and F the specimen; H, a photomultiplier tube with a quartz window; I, the viewing or monitor cathode ray tube; J, a time base which locks in time synchronism the spot scanning on the scanner tube A with the spot scanning on the monitor tube I.

Operation

The operation of the ultra-violet flying spot microscope is as follows:

Tube A is coated with an ultra-violet emitting phosphor, which, when energized by the electron beam of tube A, gives a very intense ultra-violet spot whose spectral characteristics are as given in figure II. By means of

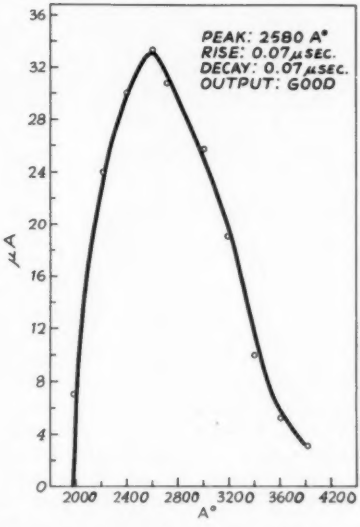

FIG. II. Spectral emission characteristics of ultra-violet scanner tube (Philco experimental tube).

time base J, this ultra-violet spot is made to transverse from the top left hand corner to the bottom right hand corner once every four seconds, tracing out in the process a television type raster of 240 lines. Simultaneously, a 240 line raster is traced on tube I in visible light; thus wherever the spot is on scanner tube A, it is in the same relative position on monitor tube I. The ultra-violet spot on scanner tube A is modified to the desired spectral form by transmission filter B, whose spectral characteristics are given by figure III. The ultra-violet transmitted by B is reflected by mirror C down through the microscope DE, and imaged onto the specimen F; *i.e.*, the specimen F is now

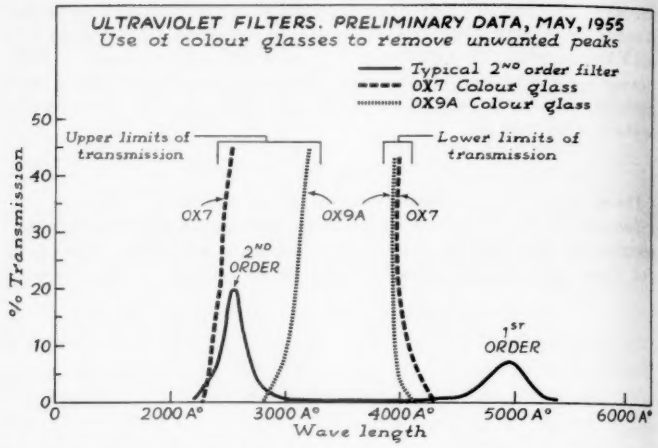

FIG. III. Characteristics of ultra-violet interference filter.

scanned by a minified spot of ultra-violet, the size of the spot tending toward ½ wavelength or approximately 0.1 micron. The amount of ultra-violet transmitted by the specimen will be determined by the spectral absorption of the specimen F, collected by condenser G, and directed by condenser G onto photomultiplier tube H. The output of the photomultiplier tube H will be a voltage varying in sympathy with the amount of ultra-violet transmitted by the specimen at any particular point in the specimen. This amplified signal voltage from the photomultiplier is now used to modulate the intensity of monitor tube I, and therefore, on the face of monitor tube I will appear a picture of the specimen delineated in terms of its ultra-violet absorption characteristics, in fact a typical ultra-violet image as is given by a standard photographic ultra-violet microscope. The slow frame scanning speed requires that a radar display tube of long persistence is required for display tube I. K is a motion picture camera which photographs the screen of monitor tube I at selected intervals which are determined by time base J, to allow four of the four second television frames to be integrated into one 16 mm. photographic frame. The film is then projected at 16 frames per second. The equipment assembly is shown in figure IV.

When the interference filter and the Corning filter are interposed, sufficient light is lost so that photographic images may only be obtained when 5 x ocular is removed from the microscope. This results in scanned field of approximately 250 millimicrons. Without the filters and with the 5 x ocular in place, the scanned microscopic field is approximately 75 millimicrons.

The cells studied were of two types. One was a strain of human fetal heart fibroblasts supplied to us by Dr. C. M. Pomerat of The University of Texas Medical Branch at Galveston, Texas. The other was the HeLa strain of

FIG. IV. Ultra-violet flying spot television equipment.

human epidermoid carcinoma cells. Both strains were grown on bare Vycor cover slips in a media of embryo extract, Gey's balanced salt solution and human ascitic fluid. They were prepared for study by mounting the cover slips and media on Vycor slides and sealing them with a paraffin-vaseline mixture. During the entire period of observation the specimens were kept at 37.5° C.

Results

With a magnification of 200 times, several cells could be scanned in one microscopic field. The spectral characteristics of the illuminating spot in this instance are given by figure III. Three separate such fields from three different preparations of fetal fibroblasts were continuously scanned and the results photographically recorded. The longest continuous period of scanning was 9 hours. In all three fields one could see actively motile cells during the entire period of continuous ultra-violet scanning. The motility consisted of two general forms. One form was the movement of broad, flattened cells by means of large flowing pseudopodia consisting of undulating membranous wave fronts. In these cases, the limiting cytoplasmic membrane was visible only when it folded upon itself. The cytoplasm of these cells showed diffuse absorption which varied according to its thickness. In the more flattened cells, the nucleus could only be discerned as an area of translucency in the cell. The intensity of the radiation here was not sufficient to clearly delineate cellular structure. The second type of motion consisted of the cell assuming a spindle shape and moving by extending long, thin pseudopodia. At the ends of these pseudopodia one could clearly make out many rapidly motile filopodia. This type of cell was much more vigorous and rapid in its motion than the former type. When the long, thin pseudopodia of these cells came into contact with those of other cells they showed a distant tendency to adhere when withdrawn. In no instance did we observe cells moving across one another. In the spindle forms of the cells the cellular detail was obscured by the intense absorption of the thickened cytoplasm. It is interesting to note that cytoplasmic absorption extended into the smallest visible radicles of the filopodia. A typical photograph of such cells is seen in figure V. All of the cells in all three specimens studied under these conditions showed continuous active unaltered mobility during the entire period of observation, and no effects attributable to the ultra-violet radiation were observed.

FIG. V. Ultra-violet absorption image of living human fetal fibroblasts. The principal absorbing materials are considered to be nucleic acids and proteins. 200x (Reproduced from a single motion picture film frame).

With a magnification of approximately 1,000 times, single, thinly spread out cells filled the major portion of the monitor screen. At this magnification the total spectral emission of the scanner tube was used to produce the image. The spectral distribution of this is given in figure II. Altogether 7 fields containing one or two such cells were continuously observed. A typical photograph of such a field is seen in figure VI. The longest such continuous period of observation was 8½ hours. In all except one instance the cells were freshly prepared from stock cultures. In this one instance, the cell observed had been in the cover slip slide preparation for three days. This cell showed many densely absorbing cytoplasmic granules approximately ½ to 2 mu. in diameter. At the end of 6 hours of continuous scan, the cell retracted its only pseudopod and then showed a sudden loss of its cytoplasmic absorption with increased nuclear membrane absorption. These phenomena were interpreted to indicate impending cell death, although the study was interrupted before nuclear opacity or cell dissolution occurred. This was the only cell in the entire study which showed any changes indicative of cellular damage. Owing to the long period which the cell was in the

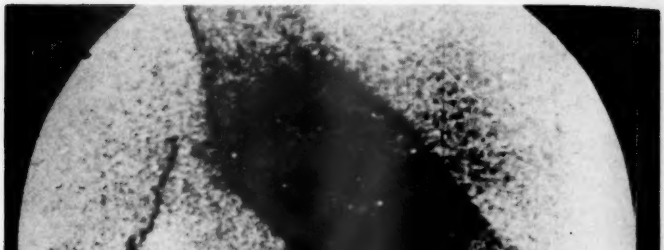

FIG. VI. Ultra-violet absorption image of living human fetal fibroblasts. The principal absorbing materials are considered to be nucleic acids and proteins, 1000x (Reproduced from a single motion picture film frame).

cover slip preparation before it was observed, it is questionable whether or not these phenomena can be entirely attributed to ultra-violet irradiation damage.

In other single cells a variety of changes in absorption and motion were observed. The typical cell showed moderate, rather general cytoplasmic absorption with frequent intensely absorbing granules. These granules were generally ½ to 1 mu. in diameter and they were clustered about the axial ends of the nuclear membrane. These granules showed continuous random motion frequently coming in and out of contact with the nuclear membrane. The absorption characteristics of these granules changed constantly. At some times they showed uniform absorption while at other times their central areas showed a distinct lack of absorption. At no time could these granules or other absorbing material be definitely seen to exchange between the nucleus and the cytoplasm. That such did not happen could not be definitely ascertained because of lack of really high optical resolution in the system. At some times perfectly round, non-absorbing vacuoles 1 to 2 mu. in diameter appeared and disappeared in the cytoplasm adjacent to the nucleus. In one instance a quite

large non-absorbing vacuole approximately 20 mu. in diameter appeared in the cytoplasm.

The cytoplasmic borders of the cell were in a constant state of activity. This consisted of broad wavelike undulations of the membrane, the extension and retraction of pseudopodia and very active pinocytosis. The cytoplasmic membrane was in general an indistinct structure. It was visible when it folded upon itself and thus increased its thickness. When a pseudopod was retracted the central absorbing cytoplasmic mass was first withdrawn into the cell leaving a non-absorbing shell extended. Along either border of this non-absorbing shell there was a thin line of absorbing material presumed to represent cytoplasm adhering to the cytoplasmic membrane. Eventually the entire pseudopod was then retracted into the cell. When active pinocytosis was occurring, it was observed to take place anywhere upon the cell surface including over the nucleus. The filopodia engaged in this very active function showed moderate absorption out to their smallest discernible endings. They appeared to be composed of fine tiny cytoplasmic extensions rather than purely membranous extensions.

The majority of the nucleus itself was translucent to ultraviolet at these wave lengths, a feature of undamaged cells described by Brumberg and Larionow (3). It appeared to be surrounded by a delicate thin line of absorbing material. This line of absorbing material was not constant in its presence, and this raises the question of whether or not a true ultra-violet absorbing nuclear membrane exists. Thus it is possible that what appears to be an absorbing membrane may in reality be due to light scatter at the interface of two different types of physico-chemical gels. When the absorbing line separating the nucleus from the cytoplasm was not visible, no movement of absorbing material from the cytoplasm entered the nucleus and no non-absorbing nuclear sap entered the cytoplasm; thus the intactness of the interface was maintained regardless of the presence or absence of an absorbing line or membrane. Some cells contained as many as three nuclei and the phenomena described applied to mono-nucleated cells as well as multi-nucleated cells.

The nuclei contained varying sizes, shapes and numbers of absorbing masses which were interpreted as nucleoli. These masses frequently changed their density of absorption. On occa-

sions they appeared almost translucent except for an absorbing, limiting membrane. As in the case of the nuclear membrane, this may have been principally due to interface light scatter and not to true membrane existence. On occasions these absorbing masses would amalgamate to form larger masses. Individual masses showed considerable motility within the nucleus and frequently came into contact with the nuclear cytoplasmic interface. No exchange of absorbing material between the nucleus and the cytoplasm was ever observed. This may have been due in a large part to the inadequacy of the resolution of the system and the obscuration of the background noise. In the HeLa cells typical nuclear rotation was observed.

Discussion

In a previous paper (2) the authors described the results of continuous ultra-violet scanning upon living HeLa cells. In this discussion it was pointed out that cytoplasmic bubbles appeared in the cells after 5 or 6 hours of continuous scanning. These bubbles were in some cases followed by marked ultra-violet absorption changes taken to indicate irreversible cell damage. The most prominent of these changes was complete lack of normal cytoplasmic absorption and homogeneous nuclear absorption. These studies were carried out utilizing quite high beam currents in the scanner tube so as to obtain the maximum illumination intensity of the phosphor. This maximum illumination was necessary because of the large light losses incident to the use of a grating monochromator after the specimen. In the present experiments no monochromator was used in the system and consequently adequate images could be obtained using far lower beam currents in the scanner tube. This in all likelihood accounts for the lack of any detectable ultra-violet damage in the present series of experiments.

Summary

1. A method for obtaining time lapse ultra-violet motion pictures of living cells has been described and illustrated.

2. Preliminary observations on the changing patterns of the ultra-violet absorption images of living cells as demonstrated by this technic have been described.

3. By the use of the flying spot technic living cells have been continuously scanned with ultra-violet light for periods up to 9 hours, with no detectable damage.

Acknowledgments

We wish to acknowledge the technical assistance of Mrs. Dorothy Robbins and to thank the Department of Medical Art for the photographic reproductions.

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[b] Electronics consultant, Department of Pathology, The University of Texas Southwestern Medical School, Dallas.

[c] Visiting scientist, Department of Pathology, The University of Texas Southwestern Medical School, Dallas.

SPECIES VARIATION IN FUNCTIONAL DISTENSION OF THE KIDNEY[a]

PETER CORNELL OVERBAUGH, M. J. RAILEY, AND H. G. SWANN

When in the dog the renal artery and vein are suddenly clamped and then the organ allowed to drain from its vein, there flows out a volume of fluid equal to some 26 per cent of the naturally distended kidney. This fluid is not blood alone, for its hematocrit is only half that of systemic blood. This, and other facts, have led to the hypothesis that the distending fluid is blood plus another fluid which we postulate to be renal interstitial fluid (Swann, Valdivia, Ormsby, and Witt, 1956). It was considered of interest to ascertain whether the same phenomenon may be observed in other species.

Methods

The amount of functional distension of the kidneys of adult mice, rats, cats and dogs was measured. Na-pentobarbitol was used as anesthetic in all cases. In mice, the kidneys were exposed with the animals in the supine position. In the other three species, the animals were suspended in the prone position by means of hooks set in the tendons and muscles of the back and skull; then their kidneys were exposed. This position permits good visualization of the organ, since the splanchnic viscera tend to fall away from it. The kidneys were entirely exposed by dissecting away all surrounding tissue except for the hilar blood vessels and the ureter. Then the artery and veins were simultaneously clamped, the ureter sectioned, and the organ removed and placed in a suitable container. The blood vessels were then sectioned between the clamp and the kidney and the organ allowed to drain for 15 minutes. The volume of the fluid draining was ascertained and the drained kidneys weighed. In the case of rat and mice kidneys, glass-stoppered, analytical weighing bottles were found suitable for this purpose. Finally, the hematocrits of the fluid which drained and of a sample of arterial blood, drawn from the animal just after nephrectomy, were measured by the microtechnic of Guest and Siler (1934). The volume of functional distension, expressed as per cent, was obtained by calculating the ratio of the volume of distending fluid times 100 to this same volume plus the weight of the drained kidney. Certain other parameters, to be described below, were also calculated.

Results

Table 1 shows the results. The number of kidneys investigated is shown, and the mean per cent distension with its standard

TABLE 1*

Natural Distension of the Kidney

	Number of Kidneys	Per cent Natural Distension		I		F
		Mean	σ	Mean	σ	
Mice	12	18.6	3.3	.86	.25	.46
Rats	18	23.0	3.6	.71	.20	.42
Cats	12	20.7	3.8	1.15	.12	.54
Dogs	50	32.2	4.1	1.04	.11	.51

* See text for meaning of symbols.

deviation (σ). Two other parameters are shown in the table, I and F:

$$I = \frac{H_b - H_k}{H_k}$$

and

$$F = \frac{H_b - H_k}{H_b} = \frac{I}{1 + I},$$

when H signifies the hematocrit and the subscripts b and k represent systemic blood and "kidney fluid," *i.e.* the fluid draining from the kidney. The ratio I, while dimensionless, actually indicates, in ml., how much volume of red cell-free fluid must be added to 1 ml. of systemic blood in order to produce a fluid with the observed hematocrit of "kidney fluid." It is, in effect, a measure of the relative volume of the postulated interstitial fluid, and it has been used to calculate the content of interstitial fluid of various substances (Swann *et al.*, 1956). The parameter F indicates the fraction of "kidney fluid" which is interstitial fluid: thus, from table 1, in the mouse, of each ml. of kidney fluid which drained, 0.46 ml. is calculated to be interstitial fluid.

The table shows that the kidneys of the three smaller species are naturally distended with a volume of fluid equal to about one-fifth of their functional volume. In the dog, however, the equivalent figure is 32.2 per cent.[b] The difference between the dog and the other three species is clear, being statistically significant at the 0.1 per cent level. The quantity of interstitial fluid, *i.e.* the quantity of cell-free fluid which is calculated to be mixed with vascular blood to produce the kidney fluid, is approximately

the same among the four species. From the ratios I or F, approximately equal volumes of blood and interstitial fluid are involved in making up the composite kidney fluid.

Discussion

It is evident that considerable functional distension of the kidney is to be found in four species of mammals. In the dog, its relative volume is about a third of the functionally distended kidney; in the mouse, rat and cat, its volume is about a fifth. The cause for this difference is not apparent: although the blood pressure greatly influences the volume of the distending fluid (Swann and Weaver, 1955), in the present experiment this is not a factor since the blood pressure of all four species is about the same (Spector, 1956).

Summary

The natural distension of the kidney in mice, rats, cats and dogs was investigated. About a fifth of the functional volume of the kidney of the first three species readily drains from the organ when the renal artery is clamped, whereas in the dog about a third drains. The fluid draining is blood mixed with another fluid which is presumed to be interstitial fluid. The latter comprises about half of the distending fluid in all 4 species.

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[a] From the Department of Physiology, The University of Texas Medical School, Galveston. This work was supported by a grant from the Life Insurance Medical Research Fund. Accepted May 15, 1957.

[b] This figure is somewhat higher than the 26 per cent distension shown for dogs in a previous paper (Swann *et al.*, 1956). The difference is due to faulty technics of dissection and drainage utilized in the earlier experiments.

THE ISOLATION AND CULTIVATION OF HEMOFLAGELLATES IN PURE CULTURE FROM SIX SPECIES OF INSECTS[a]

ARDZROONY PACKCHANIAN[b]

The natural infection of various species of insects with hemoflagellates is an established fact (Wenyon, 1926). In the past, the isolation of hemoflagellates in pure culture from the insect gut has been a rather difficult task due to other accompanying microorganisms such as bacteria, yeasts, and molds.

Novy, MacNeal and Torrey (1907) succeeded in isolating and cultivating hemoflagellates from the gut of the mosquito by inoculating blood agar slants 2 to 3 cm. above the water of condensation and with maintenance of the tubes in an almost horizontal position. This procedure, which utilized the marked motility of the flagellates over the motility of bacteria in reaching the fluid media in the bottom of the tubes, yielded good results in several instances. They were also able to obtain pure cultures by streaking the insect intestinal contents serially over six blood agar plates and subsequently selecting and isolating single colonies of flagellates on blood agar slants, usually from the last plate or two.

Nöller (1917) utilized the property of flagellate rosettes to adhere to glass surfaces for the purpose of obtaining pure cultures. By gently washing the glass surface with sterile saline, the accompanying bacteria were removed and pure cultures of flagellates were obtained on a suitable medium.

Tyzzer and Walker (1919) secured pure cultures of *Leptomonas ctenocephali* from the gut of the dog flea by means of Barber's single cell isolation technique.

Glaser (1922) inoculated the larvae of silk worms with the gut contents of the housefly containing bacteria and flagellates, and isolated a pure culture of *Herpetomonas musca domestica* directly from silk worm larvae.

Noguchi and Tilden (1926) secured pure cultures of hemoflagellates by inoculating blood agar plates with serial dilutions

of insect gut suspension and with subsequent plating until pure colonies of flagellates were obtained. They were also successful in securing cultures of hemoflagellates from acid blood agar plates, a procedure which reduces considerably the growth of bacteria. In their work with flies, they resorted to Barber's single cell micromanipulation technique for isolating flagellates. They found that suitable media implanted with several flagellates yielded isolated colonies while those implanted with single flagellates usually failed to show any growth.

Wenyon (1926) reviewed the studies of previous investigators who had succeeded in isolating and growing bacterial-free cultures of hemoflagellates from insects and added his own procedure which consisted of collecting the fecal material of blood-sucking insects such as fleas under aseptic conditions and transferring the material directly to the culture medium.

Packchanian (1934) by the use of capillary pipettes, under aseptic precautions, isolated pure cultures of *Trypanosoma cruzi* directly from the gut of *Triatoma geniculata* and *Trypanosoma melophagium* directly from *Melophagus ovinus* (sheep keds). Since both of these species of arthropods, as a rule, do not harbor bacteria' yeasts, or molds in their guts, the isolation of trypanosomes in pure culture by such a procedure was a relatively easy matter. Packchanian (1946, unpublished data) also isolated and cultured *Trypanosoma conorrhini* directly from *Rhodnius prolixus* by means of a similar technique.

The object of the present communication is to enumerate 26 species of insects which were found to be naturally infected with hemoflagellates and to present a new procedure by which hemoflagellates were isolated and cultured free from other accompanying microorganisms from the guts of six species of these insects.

Methods and Materials

A. *Collection of live insects.* Twenty-six species of live adult insects were collected during the period from 1941 to 1955 in several areas of Texas. The gut contents of 20 of these species were examined microscopically for evidence of active flagellates to determine whether they were leptomonad, crithidial or trypanosomal forms (see Table 1). The remaining six species of insects collected in Galveston County were used for the purpose of isolating and cultivating bacterial-free hemoflagellates (see Table 2).

Male and female *Oncopeltus fasciatus* (milkweed bug) were collected on many occasions almost all year around from Orlando bushes in the city of

TABLE 1

Twenty Species of Insects Found in Texas Naturally Infected with Hemoflagellates

Species of Insects Collected in Texas			Hemoflagellate Observed in the Contents of the Insect Gut	
Taxonomic Name	Date Collected and Examined	Counties of Collection	Crithidia or Trypanosome	Leptomonad or Herpetomonad
Arilus cristatus	April 8, 1949	Itasca	+	+
Brochymena parva	August 7, 1951	Galveston	—	+
Brochymena quadripustulata	August 7, 1951	Galveston	—	+
Catorhintha selector	January 8, 1941	Galveston	—	+
Chelinidea vittiges aequoris	February 24, 1941	Tilden	(?)	+
Chrysopa sp.?	July 10, 1951	Galveston	+	+
Euschistus servus	November 7, 1952	Galveston	—	+
Largus succintus	January 8, 1952	Galveston	—	+
Leptocoris trivittatus	January 8, 1952	Galveston	—	+
Leptoglossus phyllopus	September 1, 1951	Galveston	—	+
Lygaeus belfragii	April 4, 1941	Three Rivers	—	+
Lygaeus kalmii angustomarginatus	June 20, 1955	Galveston	—	+
Microlomus purcis	November 20, 1941	Three Rivers	+	+
Oncocephalus geniculatus	August 7, 1951	Galveston	—	+
Rhynchomitra microrhina	August 7, 1951	Galveston	—	+
Sinea confusa	January 28, 1941	Port Lavaca	+	+
Sinea rileyi	March 6, 1941	George West	+	+
Solubea pugnax	August 7, 1951	Galveston	—	+
Zelus cervicalis	August 7, 1951	Three Rivers	—	+
Zelus renardii	August 7, 1951	Galveston	(?)	+

TABLE 2

Isolation and Cultivation of Bacterial-Free Hemoflagellates From Six Species of Insects

Species of Insect		Cultivation of Bacterial-Free Hemoflagellates		
Taxonomic Name	Common Name	Date of Isolation	Number of Subcultures (Approximate)	Number of Days *in vitro*
Apiomerus spissipes	Squash Bug	July 7, 1951	30	1484
Musca domestica	Common House-fly	July 24, 1951	58	1467
Oncopeltus fasciatus	Milkweed Bug	August 15, 1951	32	1476
Phymata fasciata	Ambush Bug	September 1, 1951	28	1460
Tabana atratus	Horse-fly	July 10, 1951	34	1116
Zelus bilobus	"Fancy Bug"	July 6, 1951	32	1484

Galveston. In spite of the large number of adults collected, no nymphs were encountered on or around Orlando bushes.

Lygaeus kalmii angustomarginatus (plant bug), both adults and nymphs, were collected on the sidewalks of Galveston, Texas. This species of plant insects resembles *Oncopeltus fasciatus* morphologically except that it is somewhat smaller in size and adults are apparently unable to fly.

Tabana atratus (horsefly) and *Chrysops sp.* (deerfly) were collected near a barn in Galveston County and specimens of both species were found engorged with mammalian blood at the time of collection.

Specimens of *Sinea confusa* were collected in a house in George West (Live Oak County), Texas, and those of *Sinea rileyi* in a farmhouse and barn in Port Lavaca, Texas, and had presumably bitten both man and animals.

Microlomus purcis was collected in a farmhouse in Three Rivers, Texas; *Arilus cristatus* (wheel bug) was captured in the vicinity of a home in Itasca, Texas, and was said to have bitten a few people.

Remaining species of plant insects were collected in various localities, mostly around woodrat dens and cacti on ranches and farms. (See Table 1.)

B. *Method of collecting gut contents from insects for examination and cultivation of flagellates.* The gut contents of from one to three insects of each species were used in all attempts to isolate pure cultures of hemoflagellates. The abdominal and posterior areas of each insect were sterilized with one per cent lysol by means of a fine applicator. The gut contents of the insect were aspirated with a sterile Wright's pipette or tuberculin syringe containing about 0.2 ml. of Ringer's solution. The suspension was then transferred with aseptic precautions to a test tube containing 0.5 ml. of Ringer's solution. After thorough mixing, the suspension was examined microscopically. The samples which were positive for active flagellates were used as the inoculum.

C. *Method used in securing bacterial-free cultures of flagellates.* The principal culture medium used in this study consisted of two per cent infusion agar containing approximately 25 per cent defibrinated rabbit blood, a modification of the Novy and MacNeal (N.N.) medium. For each culture attempt from a given insect, about 24 blood agar slants were used, each tube containing about 0.5 ml. water of condensation. One loop or droplet of the gut suspension from each insect containing flagellates, bacteria, yeasts, molds, or combinations of two or three of these microorganisms was used as the inoculum and carefully introduced into the water of condensation of the blood agar slants without touching the slant surface of the medium. This was followed by the introduction of 3000 or 6000 mcg. of penicillin, streptomycin, or neomycin; in other tubes a combination of these three antibiotics, 3000 or 6000 mcg. of each, was used per N.N. tube. Duplicate tubes were used for each concentration. In the isolation of flagellates from *Phymata fasciata*, aureomycin was substituted for neomycin.

In a few instances, in order to eliminate growth of certain yeasts and molds which were found resistant to these antibiotics, media consisting of blood agar containing 0.005 per cent of copper sulfate, or 0.5 per cent of sodium benzoate, or 0.5 per cent of calcium propionate were used. Endo's medium enriched with 20 per cent defibrinated rabbit blood was utilized in some cases.

All culture tubes were rubber capped and incubated in an upright position at 25° C. for two to four weeks before they were examined, macroscopically and microscopically, for evidence of growth of flagellates. The tubes showing bacterial growth were discarded and those which showed pure cultures of flagellates were subcultured every two to four weeks on blood agar slants for several generations without further addition of antibiotics or chemicals.

One representative culture of hemoflagellates isolated from each species of insect and showing luxuriant growth free from other microorganisms was selected as stock culture. These stock cultures were transferred monthly or bimonthly on blood agar slants without further addition of antibiotics or chemicals and have been maintained *in vitro*, from four to over five years (see Table 2). A few of these stock cultures were inoculated on blood agar plates for colony observation and some were used for fermentation, serologic, and immunochemical studies.

Experimental Data

The results of these studies are summarized in Tables 1 and 2 and can be briefly described as follows:

A. NATURAL INFECTION OF INSECTS WITH HEMOFLAGELLATES. Twenty-six species of insects collected in Texas were found to harbor hemoflagellates in their guts. Of these, 20 species were naturally infected with leptomonads and six with trypanosomes, the majority of the latter group were in the crithidial form. Trypanosomes with complete but narrow undulating membrane were noted in *Arilus cristatus*, *Microlomus purcis*, *Sinea confusa* and *Sinea rileyi*. Broad crithidial forms of active flagellates were encountered in *Chrysops sp.* and *Tabana atratus*. The microscopic examination of the gut contents of the remaining species revealed only typical leptomonad forms.

No special efforts were made to determine the various species of flagellates seen or isolated from these insects other than to differentiate them as morphologically leptomonad, crithidial, or trypanosome in form. (See Tables 1 and 2.)

B. ISOLATION AND CULTIVATION OF HEMOFLAGELLATES. The pure cultures of hemoflagellates were isolated from six species of insects as follows:

1. *Isolation of leptomonads in pure culture from* Apiomerus spissipes (*squash bug*). In three experiments, five positive cultures of bacterial-free flagellates were obtained out of 19 blood agar slants (N.N. tubes) containing 3,000 or 6,000 mcg. of penicillin, streptomycin or neomycin, or 3,000 mcg. of each of the three antibiotics combined. Of the five positive cultures, two

were found in N.N. tubes containing 6,000 mcg. of streptomycin or neomycin and three in tubes containing 3,000 mcg. of each of the three antibiotics combined.

The remaining 14 tubes which contained 3,000 mcg. of individual antibiotics showed heavy growths of bacteria, yeasts or molds with occasionally a few sluggish flagellates. All of these tubes were discarded without any further attempt to isolate the flagellates from accompanying microorganisms.

The leptomonads isolated in pure culture from *Apiomerus spissipes* grew luxuriantly on N.N. media containing 0.005 per cent of copper sulphate, or 0.5 per cent of calcium propionate or 0.5 per cent of sodium benzoate. Endo's medium enriched with 20 per cent defibrinated rabbit blood and both liquid and semi-solid leptospira media also supported the growth of flagellates.

Growth of colonies on blood agar plates after nine days incubation at 25° C. were non-hemolytic, measured approximately 2 to 4 mm. in diameter, appeared moist, circular, slightly convex, smooth, glistening, erose, radiately ridged and concentrically ringed, consisting of four zones. On calcium propionate blood agar plates, the colonies were thin, spreading, translucent, flat, irregular and non-hemolytic. On chocolate blood agar, colonies were gray, with a diameter of 2 to 3 mm., smooth, raised, glistening, entire and non-hemolytic.

2. *Isolation of leptomonads, "herpetomonads," in pure culture from* Musca domestica (*house fly*). From four N.N. tubes containing 6,000 mcg. of penicillin, streptomycin or neomycin, or 6,000 mcg. of each of the three in combination, two positive cultures were secured. The first was obtained in the N.N. tube containing 6,000 mcg. of the three antibiotics in combination, the second in the tube containing 6,000 mcg. of neomycin.

Another set of four N.N. tubes identical to those used above with respect to type of antibiotics but containing in addition 0.005 per cent of copper sulphate yielded two positive cultures of flagellates free from other microorganisms. One of these positive cultures occurred in the tube containing 6,000 mcg. of penicillin and the other in the tube containing 6,000 mcg. of each of the three antibiotics in combination.

The remaining culture tubes which contained 3,000 mcg. of antibiotics or chemicals failed to eliminate the growth of bacteria, yeasts, and molds. All these tubes were discarded without fur-

ther attempts to isolate the flagellates from accompanying microorganisms.

The four successful bacterial-free cultures of *Leptomonas musca domestica*[c] isolated were subcultured monthly or bimonthly on blood agar slants without further addition of antibiotics for many generations. One of these strains was selected as stock culture and has been maintained *in vitro* for over five years.

Leptomonas musca domestica were grown in large quantities in liquid medium, and from such growths 10 g. of washed acetone-killed dry powder of these flagellates were collected for immunochemical studies.

3. *Isolation of leptomonads in pure culture from* Oncopeltus fasciatus (*milkweed bug*). Four culture tubes out of 25 yielded bacterial-free cultures of flagellates; however, these were associated with yeasts. Two of these positive findings occurred in tubes containing 6,000 mcg. of streptomycin or 6,000 mcg. of each of the three antibiotics combined and two were obtained in duplicate tubes containing 0.5 per cent of calcium propionate. Although in the initial isolation yeast colonies were present on the slant surface of the medium along with the flagellate colonies, careful isolation of flagellates on subculture resulted in pure cultures of leptomonads. A strain of leptomonad has been maintained *in vitro* on N.N. agar by monthly subculture for several years. These readily infect laboratory-reared, normal *Oncopeltus fasciatus*.

4. *Isolation of leptomonads in pure culture from* Phymata fasciata (*ambush bug*). A series of four N.N. tubes containing 6,000 mcg. of penicillin, streptomycin, or aureomycin or 6,000 mcg. of each of the three in combination and inoculated with a suspension of the gut contents of one insect yielded four positive cultures of flagellates free from accompanying microorganisms. Three additional positive cultures of flagellates free from bacteria were obtained in N.N. tubes containing 6,000 mcg. penicillin and 0.5 per cent of sodium benzoate, in tubes containing 6,000 mcg. of aureomycin and 0.005 per cent of copper sulphate, and in Endo-blood medium containing 6,000 mcg. of streptomycin. These positive cultures of flagellates were subcultured on N.N. medium for several generations without further addition of antibiotics. They grew luxuriantly, not only in the water of conden-

sation of the medium, but also on the slant surface of the N.N. tubes.

5. *Isolation of crithidia in pure culture from* Tabana atratus (*horsefly*). Twenty-seven N.N. tubes containing 6,000 mcg. or 1,200 mcg. of penicillin, streptomycin or neomycin and 6,000 or 8,000 mcg. of each of the three antibiotics combined which were inoculated with suspensions of the gut contents of three insects in three experiments yielded nine positive cultures of flagellates free from other microorganisms. Five of these occurred in tubes containing 6,000 or 8,000 mcg. of streptomycin.

The morphology and biology of hemoflagellates from *Tabana atratus* both in the initial smear of the insect gut and in cultures were indistinguishable from the cultural form of *Trypanosoma theileri* isolated from cows several years ago (Packchanian, 1934).

6. *Isolation of leptomonads in pure culture from* Zelus bilobus ("*fancy bug*"). Seven N.N. tubes containing 3,000 or 6,000 mcg. of penicillin, streptomycin, or neomycin or 3,000 mcg. of each of the three antibiotics combined which were inoculated with a suspension of the intestinal contents of one insect yielded one positive culture of flagellates free from bacteria. This occurred in the N.N. tube containing 6,000 mcg. of neomycin.

In another experiment, the suspension of insect gut material was exposed to 3,000 mcg. of each of the three antibiotics combined for periods of from 1 to 24 hours and inoculated into N.N. tubes. This yielded two positive cultures of bacterial-free flagellates.

The leptomonads isolated in pure culture from *Zelus bilobus* also grew readily on N.N. media containing 0.005 per cent of copper sulphate or 0.5 per cent of calcium propionate or 0.5 per cent of sodium benzoate and on Endo's medium enriched with 20 per cent defibrinated rabbit blood.

After nine days of growth on blood agar plates, single colonies measured 2 to 4 mm. in diameter; they were moist, circular, slightly convex, smooth, glistening, non-hemolytic, erose, radiately ridged and concentrically ringed, consisting of four zones.

On calcium propionate blood agar plates, single colonies measured about 6 mm. in diameter; they were dull gray, smooth, raised, glistening, entire, and non-hemolytic. Spreading colony growth was thin, translucent, flat, irregular and non-hemolytic.

On chocolate blood agar, colonies were small, measuring 2 to 3 mm. in diameter. They were gray, smooth, raised, glistening, entire, and non-hemolytic.

The flagellates grew readily on simple liquid medium, such as beef extract broth, hay infusion broth, potato-water broth, proteose phosphate broth, and tryptose broth, to which minute quantities of rabbit serum were added (one drop of serum per 10 ml. of medium). There was no growth on hay infusion broth to which no serum was added.

Leptomonads (isolated from *Zelus bilobus*) grew on arabinose, dextrin, dulcitol, inositol, lactose, rhamnose, sorbitol, and xylose without fermenting any of these carbohydrates. Growth with production of acid but no gas occurred in galactose, glucose, inulin, maltose, mannitol, raffinose, salicin and sucrose.

These flagellates were grown in large quantities, and washed flagellates inoculated into rabbits intravenously three times a week yielded antibodies which agglutinated homologous antigen in a titer of over 1:10,000.

C. Stock cultures of hemoflagellates isolated from the six species of insects. Representative stock cultures of flagellates selected from the six species of insects have been kept *in vitro* on blood agar slants for from 1,116 to 1,484 days and subcultured monthly or bimonthly from 28 to over 58 times. (See Table 2.)

Stock cultures grew luxuriantly, not only in the water of condensation of the N.N. medium, but also on the slant surface. The viability of these flagellates on N.N. tubes is rather long. With the exception of the trypanosome isolated from *Tabana atratus* which required monthly transfer, the stock cultures from the remaining five species of insects were invariably active and always yielded positive subcultures at the end of two months. At times, subcultures were obtained from stock culture tubes which were six months old and in a few instances from tubes that were as much as two years old. The stock cultures have been examined from time to time and it was noted that all flagellates isolated from the six species of insects have retained their original morphological characteristics throughout all subcultures.

Discussion

Although it is possible to isolate certain flagellates from mixtures with bacteria by the original plating method used by Novy

or by Barber's single cell isolation technique and by the use of various motility and aerotropic properties of flagellates used by various investigators, these procedures are rather laborious.

The remarkable selective antagonistic effect of certain antibiotics against bacteria without harmful effect to flagellates made possible the isolation and cultivation of six species of flagellates free from bacteria as described in this paper. The procedure described is also useful in repurifying flagellate cultures accidentally contaminated with bacteria and will facilitate ready cultivation of *Leishmania tropica* in pure culture from oriental sores containing many bacteria. (Packchanian, unpublished data, 1947.)

In attempts to cultivate bacterial-free flagellates from insects by the procedure described in this paper, yeasts and molds presented some difficulty because of the ineffectiveness of the antibiotics used against them. Further studies are in progress in order to develop a selective culture medium which will inhibit the growth of yeasts, molds, and bacteria and yet favor the growth of flagellates in pure culture.

Summary

1. Of the twenty-six species of insects collected in various localities of Texas, 20 species were found to be naturally infected with leptomonads and six with trypanosomes.

2. A new procedure is described by which hemoflagellates were cultured free from accompanying bacteria from the insect gut by the addition of adequate concentrations of neomycin, penicillin or streptomycin, individually, or in combination together with the inoculum into the water of condensation of the slightly modified N.N. medium. These antibiotics in appropriate concentration apparently served as bactericides or bacteriostatics and facilitated the luxuriant growth of the flagellates in pure culture.

Hemoflagellates were isolated and grown in pure culture from the following six species of insects: *Apiomerus spissipes*, *Musca domestica*, *Oncopeltus fasciatus*, *Phymata fasciata*, *Tabana atratus*, and *Zelus bilobus*.

3. The flagellates isolated from *Tabana atratus* were crithidial in form and had all the morphologic characteristics of culture forms of *Trypanosoma theileri*. The flagellates isolated from the remaining five species of insects all had the morphol-

ogy of leptomonads; no crithidial or trypanosomal forms were noted in the initial smears nor in subcultures from this group.

4. Rabbits were immunized with washed cultures of leptomonads isolated from *Zelus bilobus* and an antiserum with high agglutinin titer against the homologous antigen was obbtained. Ten grams of washed acetone-killed dry powder of *Leptomonas musca domestica* was collected for immunochemical studies.

5. Bacterial-free stock cultures of the flagellates isolated from the six species of insects have been maintained *in vitro* on blood agar slants with monthly or bimonthly transfers for periods of from four to five years. These flagellates grew luxuriantly not only in the water of condensation but also on the slant portion of the medium and retained their original morphologic characteristics throughout all subcultures.

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[b] Department of Bacteriology and Parasitology and Laboratory of Microbiology, School of Medicine, The University of Texas, Galveston, Texas.

The writer wishes to express his thanks to Miss Bettie Celaya, and Miss Rose Tennant for their valuable assistance and interest in this study.

[c] In the literature the flagellate in house fly is referred to as *Herpetomonad musca domestica* which in the opinion of the writer is a misnomer.

EFFECT OF ELECTRO-SHOCK ON THE NEUROHYPOPHYSIS OF THE RAT[a]

EDWARD G. RENNELS AND GLENN V. RUSSELL[b]

It is generally recognized that a wide variety of stimuli will lead to a discharge of antidiuretic hormone (ADH) as measured by increased titres of ADH (Verney, 1947). It has also been reported that painful stimuli such as pricking the tail will lead to a rapid decrease in the amount of stainable neurosecretory material in the neurohypophysis of the rat (Rothballer, 1953). It is now generally held that this stainable material is intimately associated with the posterior lobe hormones (Scharrer and Scharrer, 1954) and that both are produced by neurones of the supraoptic and paraventricular hypothalamic nuclei. It seemed of interest, therefore, to subject animals to a standard stress and to study the content of neurosecretion and of hormones in the posterior lobe at increasing time periods after stress. Preliminary experiments had failed to confirm Rothballer's findings with respect to neurosecretory changes after pricking the tail so it was thought that a more severe stress such as can be induced by electro-shock should be used. The results indicate that a significant discharge of these materials does not follow this particular type of stress in the time periods tested.

Materials and Methods

Animals. Young male rats of the Sprague-Dawley strain weighing approximately 225 grams were used in this study. A total of 124 animals were used, 72 for the purpose of supplying pituitary glands for hormone assays and 52 for histological study of their pituitary glands. All animals were killed by decapitation. The control animals received no treatment, whereas all others were subjected to electro-shock supplied by ear-to-ear leads. When the ear clips were secured, a 45 milliamp, 18 volt DC stimulus was administered for 10 seconds. This produced a generalized tonic seizure which lasted for the duration of the stimulus and which was followed by a prolonged period of lethargy. The rats were usually shocked and killed in groups of 20. The different groups studied with reference to the interval after shock when the animals were killed are indicated in table 1. At the time of decapitation the brain was removed rapidly and a block of hypothalamus about 3 mm. in each dimension was cut out with a razor blade. This was placed either in acetone

when the hormone content was to be assayed or in Maximow's fixative when histological studies were to be carried out. Immediately after this the pituitary gland was removed and handled in a similar manner. The maximum time required for this procedure from decapitation until hypothalamus and hypophysis were in the appropriate solution was 90 seconds. It should be noted that in table 1 the animals in the group designated "0 min." were decapitated immediately after the standard 10 second period of shock.

TABLE 1

Time after shock	Animals for Hormone Assays		Animals for Neurosecretion Studies	
	Groups	Total No.	Groups	Total No.
No shock	4	17	3	10
0 min.	1	4	2	8
3 min.	1	4	3	10
15 min.	1	4	3	10
30 min.	4	17	3	10
1 hour	3	13	1	2
3 hours	3	13	1	2

Assay Methods. Hypothalami and pituitary glands to be assayed for hormone content were dehydrated in three changes of acetone and stored in a dessicator. Each group of 4 or 5 pituitary glands was ground in a mortar and extracted with 3 ml. of boiling 0.25 per cent acetic acid for 3 minutes. This suspension was filtered and the filtrate kept frozen until such time as it was assayed.

The content of oxytocin was determined by use of a modification of the method described by Coon (1939) which takes advantage of the depressor effect exerted by oxytocin on the blood pressure of the rooster.[e] Rhode Island Red birds weighing 1.8–3.0 kg. were anestetized by an intravenous injection of 200 mgm./kg. of sodium phenobarbital and prepared for recording blood pressure by cannulation of the ischiadic artery. Blood pressure was recorded with a mercury manometer with a float operating the recording pointer on a slow moving kymograph. Injections were made into the crural vein. Each determination was based on a series of 10 injections, six of these were standard preparations of Pitocin (Parke, Davis and Co.) and 4 were of the extract being assayed. Three dose levels of the standard were used, such that S_3 was 3 times S_1 and 2 times S_2. Two dose levels of the unknown extract were used with U_2 being double the volume of U_1. Each dose level was repeated once in the course of a determination. The order of injections used was S_2, U_1, S_3, U_2, S_1, S_2, U_1, S_3, U_2, S_1. Measurements were made of the depression of blood pressure in millimeters, produced by each injection and the average of the two values for each of the three dose levels of Pitocin were plotted on semi-log graph paper. This regression line was then used in obtaining the hormone content of the unknown extract in terms of milliunits of the Pitocin standard. It was found that 3 or 4 determinations could usually be obtained from a given rooster before the responses were so reduced by tachyphylaxis as to be unreliable. The major disadvantage of this assay method is that it is relatively

insensitive and in the present study the number of oxytocin assays performed on some of the extracts is consequently rather small.

It should be noted that some oxytocin assays were performed on hypothalamic extracts but this was soon discontinued because it was found that relatively little activity was present and furthermore that extracts of other areas of the brain such as cerebral cortex and cerebellum contained about the same amount of activity.

A pressor assay similar to that described by Landgrebe, *et al.* (1946) was used for measuring the vasopressor content of the pituitary extracts. In these assays we used large male rats weighing in excess of 400 gms. and the initial anesthesia was provided by an intraperitoneal injection of 6 mgm. sodium pentobarbital (Nembutal) per 100 gm. body weight. The trachea is cannulated and respiration maintained by a mechanical respirator during the assay. Prior to cannulating the left common carotid for recording blood pressure, 2 mgm. of heparin sodium in gelatin (Upjohn) is injected intravenously. The carotid cannula is connected to a mercury manometer of 2 mm. bore and blood pressure recorded on a kymograph. The femoral vein is then exposed and a 26 gauge hypodermic needle secured in the vein. At this time, deep anesthesia is obtained by giving more Nembutal, 6 mgm. intravenously followed by 18 mgm. subcutaneously. The initial blood pressure is usually about 150 mm. Hg. This is somewhat reduced by Nembutal and at this time hexamethonium chloride is administered, 5 mgm. intravenously and 12.5 mgm. subcutaneously. This ganglion blocking agent reduces the blood pressure promptly to a base line of about 50 mm. and the animal is ready for use. In this study each determination is based on a series of 5 responses, 3 of which were to three dose levels of a standard preparation (Pitressin—Parke, Davis and Co.) and two of which were to 2 dose levels of the unknown. The doses of the unknown should be such that both unknown responses fall between the low and high response to the standards. The log dose response line is then plotted and the value in units of the unknown response determined. There is a prompt return of blood pressure to the base line following each injection so that injections can be made at intervals of 3–5 minutes. Several series of injections can be made on each rat preparation and when the activity of different extracts are to be compared, as in the present study, several can be assayed on the same rat.

For each group of animals listed in table 1 under "Animals for Hormone Assays" an extract was prepared and assayed in three or more rats.

Histological Methods. The pituitary glands were fixed overnight in Maximow fluid, washed in running water, dehydrated in an alcohol series and cleared in cedarwood oil. After paraffin imbedding, they were sectioned at 5 micra and mounted serially. They were stained with the chrome-hematoxylin-phloxine method described by Gomori (1941). This method gives a selective coloration to the neurosecretory material of the posterior lobe.

Results

The values obtained for the content of oxytocin and vasopressin in the pituitary gland of normal male rates are given in fig-

ures 1 and 2. All values are expressed in milliunits of activity per pituitary gland. The mean value found for oxytocin was 366 ± 21, and that for the vasopressin was 278 ± 12. At 3 minutes and 15 minutes after electro-shock the content of oxytocin was somewhat increased although these differences are not of statistical significance at the 5 per cent level. The pituitary content of oxytocin was not significantly reduced by electro-shock at any of the times tested.

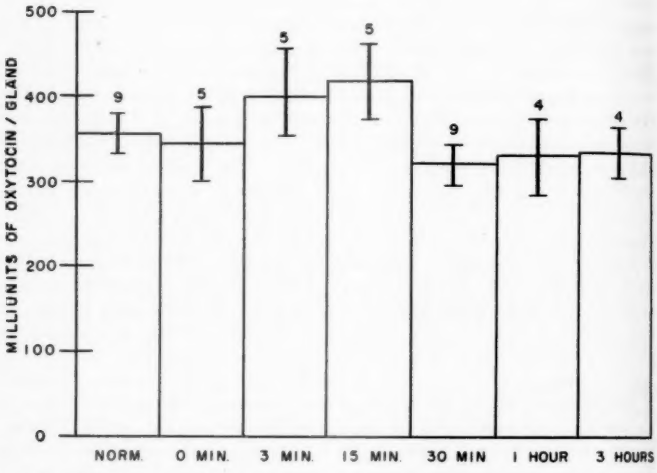

Fig. 1. Effect of electro-shock on pituitary content of oxytocin (mU / gland) in normal rats (NORM.) and at the time intervals indicated following a standard electro-shock. Columns represent means and standard errors with the number of assays given above each column.

The content of vasopressin in the pituitary gland after shock was increased at 15 minutes and again at 3 hours. At 30 minutes and 1 hour it was very little different from the control level. A significant reduction in the pituitary content of vasopressin was not found, although it must be admitted that there are deficiencies in the assay data, particularly in the number of glands assayed at short time intervals after shock.

The neurosecretory material of the posterior lobe is well shown by Gomori's chrome-hematoxylin-phloxine method and has been sufficiently well illustrated in the recent literature. All

areas of the glands were examined in this study and no features were observed which could be considered as diagnostic of the glands from the shocked animals. It is certain that no marked alteration in the content of neurosecretion was seen in any of the glands that could be attributed to the electro-shock. It is equally certain that no alterations in the vascularity of the posterior lobe, such as those reported by Rothballer (1953) were observed. Neurosecretory material within the vessels was not seen

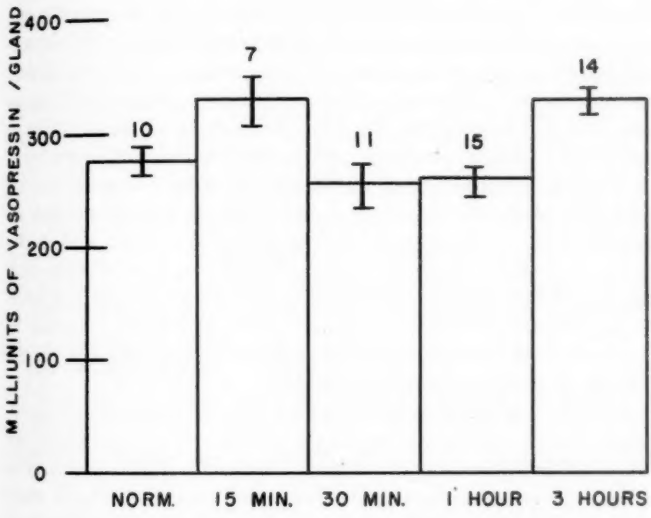

Fig. 2. Effect of electro-shock on pituitary content of vassopressin (mU/gland) in normal rats (Norm) and at the time intervals indicated following a standard electro-shock. Columns represent means and standard errors with the number of assays given above each column.

in any of the glands and no evidence of a discharge of neurosecretory material was observed. Suffice it to say, that no satisfactory method was found for separating the glands of the shocked animals from those of the controls other than that of referring to the record book.

Discussion

The control levels obtained for the pituitary content of oxytocin and vasopressin are in very good agreement with the control values reported by Dicker and Tyler (1953) for the rat. In both cases the V/O ratio is close to unity.

It seems reasonably well established that noxious stimuli of various types will elicit a discharge of ADH from the neurohypophysis. It also appears that ADH activity is a property of purified vasopressin (Popenoe, *et al.*, 1952). It was somewhat surprising, therefore, to find that no significant decrease in the pituitary content of vasopressin occurred following the electro-shock used in the present study. It was even more surprising that an increase in the content of this hormone was found at 15 minutes and at 3 hours after the shock. We attach no importance to these latter changes, however, since they are difficult to interpret in a rational manner in terms of what is known about posterior lobe physiology. Furthermore, it is felt that in view of the known sources of error inherent in the method of extraction and in the bioassay method, the results of significance tests applied to such data should be viewed with some caution. In this regard, it must be kept in mind that the numbers given above the columns in figures 1 and 2 refer to rat or rooster assays and not to the number of pituitary glands assayed.

It is of some interest that Cavalca (1952) found that electro-shock in rats caused an increased diuretic response to a water load and decreased the antidiuretic property of the serum. The shock administered by Cavalca was a 70-volt stimulus of short duration (.2–.3 sec.) which produced a tonico-clonic convulsion. This suggests that electro-shock may give quite a different response than that considered typical of an emotional or painful stress reaction.

The pituitary content of oxytocin was similarly not reduced by electro-shock. Some slight increase was observed at 3 and 15 minutes after shock. Again we attach no great importance to these minor alterations. It was felt that the electro-shock used would constitute a severe stress but this point may be subject to some debate since the animals were quiescent and in a state of subdued activity following the 10 second stimulus. At any rate, it is clear that no significant reduction in either vasopressin or

oxytocin occurred. It is equally clear that no alteration in neurosecretion resulted from the electro-shock treatment. It seems paradoxical that minor emotional or painful stimuli can result in a marked depletion of this material as reported by Rothballer (1953) and that an electro-shock resulting in a generalized tonic seizure will fail to stimulate posterior lobe discharge. Such, however, appears to be the case.

Summary

Rats were subjected to an electric shock of 45 milliamps, 18 volts DC for 10 seconds. Following this shock, groups of animals were killed at intervals of time up to 3 hours. The pituitary glands of these animals were assayed for their content of oxytocin and vasopressin. Other glands were fixed and stained with Gomori's chrome-hematoxylin-phloxine stain for neurosecretion. It was found that no discharge of posterior lobe hormones occurred in the time period tested and that the neurosecretory material of the posterior pituitary gland was unaltered.

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[a] This study was supported by Research Grant B-410 from the U. S. Public Health Service. Accepted for publication May 20, 1957.

[b] From the Department of Anatomy, The University of Texas Medical Branch, Galveston, Texas.

[c] Assay method adopted by U.S.P. and B.P. for Oxytocin.

PROCEEDINGS OF THE TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Abstracts of Some of the Papers Read at the Spring Meeting

March 29–30, 1957

The University of Texas Medical Branch
Galveston, Texas

The Use of Hexadecanol as a Carbon Source for Certain Bacterial Species. R. K. Guthrie and Nelda Hinckley, North Texas State College, Denton, Texas.

Water samples from two reservoirs were tested to determine the number of *Pseudomonas* and *Alcaligenes* organisms present. Two to seven colonies of each organism were present. One reservoir was then treated with cetyl alcohol (hexadecanol), and about four weeks later samples from both reservoirs showed that the *Pseudomonas* had increased to 57 colonies, *Alcaligenes* to 28 in the treated reservoir. Counts of both organisms remained constant in the untreated reservoir. *Pseudomonas* and *Alcaligenes* strains isolated from water, were tested in Frazier-Rupps mineral solution containing only hexadecanol as a carbon source. Twenty-seven strains of these organisms grew in the presence of hexadecanol, indicating that these organisms can and do degrade this evaporation control agent.

The Effect of Variously Treated Guinea Pig Sera on the Survival Time of Akr Mice Bearing Transplanted Lymphoid Leukemia. George K. Schlagenhauf, R. K. Guthrie and D. M. Morris, North Texas State College, Denton, Texas.

The survival time of Akr mice after an intraperitoneal injection of a suspension of tumor cells from leukemic mice of the same strain was increased by a single intraperitoneal injection of 0.2 cc. of anti-leukemic tissue guinea pig serum given at the time of the implant. Similar injections of this serum in which the C'3 component of complement had been inactivated, increased

the survival time to the same extent, while C′4 inactivated serum offered no apparent protection. Normal guinea pig serum and typhoid-immunized guinea pig serum injected as above had no effect upon the survival time of such mice.

Reduction of Coliform Bacteria in Sewage Sludge by Chlorination. C. H. Connell, Donald A. Dreyer and E. J. M. Berg, Department of Preventive Medicine and Public Health, The University of Texas Medical Branch, Galveston, Texas.

The most probable numbers of coliform bacteria/100 ml. (MPN) in homogenized non-chlorinated sludge has ranged from 10^8 to 10^9. Chlorination of non-homogenized samples to 2-hour residuals of 20 to 60 mg./l reduced MPN only to the range of 10^4 to 10^6. Two-hour chlorine residuals of 30 to 60 mg./l in samples that had been homogenized consistently reduced MPN to below 100/100 ml. Two-hour residuals as low as 10 mg./l in homogenized samples reduced MPN to below 1000/100 ml. These results demonstrate that the numbers of coliform bacteria in fresh sewage sludge can be reduced to very low values by readily attainable chlorine residuals when the solids are dispersed by reasonably short periods of homogenization.

Loss of Photoreversal Effects During Sporulation. W. R. Romig and Orville Wyss, The University of Texas, Austin, Texas.

Endotrophically sporulating cultures of *Bacillus cereus* were shown to gain the ultraviolet (UV) resistance characteristic of mature spores more than one hour before heat resistance is manifested. The increased UV resistance of the sporulating culture coincides with the loss of susceptibility to photoreactivation, and cytological studies show these changes in the photoresponse of the culture to be associated with the appearance of the forespore. Low doses of UV cause an 8 hour lag in the appearance of spores in the surviving population and greatly reduce sporulation in the survivors. This UV-induced lag can be partially photoreversed both as to the delay in onset and the extent of sporulation. The UV damage to the spore-forming mechanism is most severe during the early stages of sporulation and by the time forespores are formed, low UV doses are not sufficient to appreciably inhibit spore formation.

The Relationship Between Coagulase Production and Various Biochemical Properties of "Micrococcus pyrogenes" var. "aureus and albus." Judith Marston and W. J. Fahlberg, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

Coagulase production and other biochemical activities of strains of staphylococci have been associated with the possible pathogenicity of these strains.

This study was undertaken to correlate, if possible, some of these activities and coagulase production. Growth on Tellurite-Glycine agar, mannitol fermentation, delta and beta lysin production, urease production, pigmentation and coagulase production were studied.

Excellent correlation between coagulase production and growth on tellurite-glycine agar was obtained. There was equivocal correlation with the other biochemical activities, by either *aureus* or *albus* strains under the conditions of the experiment.

Frequency of Occurrence of Various Organisms in the Anterior Nares of a Hospital Population. Joe A. Bass, Warren R. Stinebring, and Harriet M. Felton, The University of Texas Medical Branch, Galveston, Texas.

A survey of the anterior nasal flora of all personnel associated with the John Sealy Hospitals showed a relatively high frequency of occurrence of pseudomonas, proteus, and other gram negative species. Although incidence of these organisms, as well as *S. aureus*, was high in patients, their occurrence in other groups could not be related to their proximity to the patients. On the other hand, distribution of phage type 81 *S. aureus*, known to be responsible for recent outbreaks of staphylococcal infections in patients and personnel, while not in proportion to the occurrence of *S. aureus* strains in general, was clearly related to proximity of any given group to the patients.

Effect of Dextrose on Lysis of Staphylococcus by Phage. The University of Texas Medical Branch, Galveston, Texas. A. Ruysenaars.

Addition of dextrose to a broth in quantities of 0.2 to 1.0 per cent had a complete inhibitory effect on the lysis of staphylococcus organisms by homologous bacteriophage.

This was partly but not altogether due to pH, the reactions changing from initial 7.4 to 5.2. Lysis of staphylococcus by phage in dextrose free acid broths was incomplete and took much longer than in non-acid broths. When dextrose broths were buffered lysis took place, but completely so only in tubes containing low host cell and high phage suspensions.

From the incompletely lysed tubes phage resistant staphylococcus was recovered. These appeared to have developed capsular material.

Plaque counts indicate that replication takes place in the presence of dextrose, but fewer plaques form and the majority are smaller in size. It is believed that both dextrose and the acid formed operate in lysis inhibition.

Identification of Feline Pneumonitis Virus in Tissue Culture by Fluorescein Labelled Antibody. P. Donaldson and S. E. Sulkin, The University of Texas Southwestern Medical School, Dallas.

Methods for early detection and recognition of a member of the psittacosis lymphogranuloma venereum group were sought. Cat anti-feline pneumonitis convalescent sera were used to sensitize feline pneumonitis antigen in infected yolk sac sections and yolk sac tissue cultures. Specific staining of sites of the infection then occurred as a result of treatment with rabbit anti-cat globulin conjugated with fluorescein. Specific fluorescent staining could be seen in yolk sac sections following 21 hours infection with about 10^5 ID. Earliest specific staining so far observed with yolk sac culture was at thirty hours.

Growth of Meningopneumonitis Virus in Normal and Immune Monocytes. Albert A. Benedict and Clarence McFarland, The University of Texas Medical Branch, Galveston, Texas.

Meningopneumonitis virus proliferated in cultures which consisted of 10^6 guinea pig monocytes (peritoneal exudates) and 30 per cent homologous serum in Hank's solution. Infectious virus was detected throughout the latent period (12–24 hrs.), followed by increased extracellular and intracellular virus titers. Approximately 50 per cent of noninfected and infected monocytes degenerated within the 24 hours, and thereafter a stable cell population was maintained up to 120 hours. Virus prolifer-

ated without obvious cytopathogenic effects or decreased monocyte counts. Monocytes obtained from psittacosis-infected guinea pigs, and in the presence of normal serum, suppressed virus growth. Growth in immune cells was a function of the infecting inoculum concentration. Only partial suppression occurred with high infecting inocula (LD_{50} of 5.70).

Some Characteristics of the Salivary Gland Virus. Alice Conklin.

Salivary Gland virus is found only in salivary glands of bats, never brains, in no other tissue. Mice inoculated intracranially have the virus in salivary glands as well as brain. Thus far, this virus visibly infects only white mice. Mice may be infected intranasally. Ether solubility was reported.

Viremia was studied by intracutaneous inoculation. Virus was recovered from the blood of guinea pigs and mice at twenty-four, forty, and forty-eight hours, not thereafter. Also from the salivary glands of mice at 96 hours.

Antibody levels against Salivary Gland virus were studied in sera of laboratory personnel, by complement fixation and neutralization tests. Personnel handling *T. mexicana* showed both complement fixing and neutralizing titers. There was good correlation between these titers. Personnel not handling bats showed none.

Three of thirty-six individual bat sera were found to neutralize 1,000 mouse LD/50 virus.

Viral Inactivation by Heat-Labile Serum Factors. Rae Allen, Richard A. Finkelstein and S. Edward Sulkin, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

Examination of viruses for susceptibility to heat-labile inhibitors in normal rat serum has revealed a range of reactions including complete resistance (Lansing poliovirus, Adenovirus Type 4), moderate resistance (St. Louis Encephalitis, Murray Valley Encephalitis) and marked susceptibility (Herpes). Susceptibility was determined by titrations (in HeLa cells or mice) of virus incubated at 37° C. for one hour in either fresh or heated (56° C./30 minutes) pooled rat serum. Participation of the properdin system in inactivation of herpes virus by normal

serum *in vitro* was implied by the marked reduction in inhibitory titer of serum treated with zymosan which removes properdin. Resistance of herpes virus, initially inhibitor-sensitive, has been increased by selection of survivors after treatment with inhibitor.

Passive Hemagglutination Method for Detection of Psittacosis Antibody. Edith O'Brien and Albert A. Benedict, The University of Texas Medical Branch, Galveston, Texas.

A passive hemagglutination test for the psittacosis-LGV group of viruses was described. After removal of virus from meningopneumonitis-infected allantoic fluids, a soluble group antigen was sedimented from the fluids by centrifugation at 100.000 x g for 80 minutes. This antigen was employed for sensitization of tanned erythrocytes. The sensitizing antigen and the murine erythrocyte hemagglutinin appeared to represent different antigenic moieties. Serologically active substances which were responsible for non-specific hemagglutination by normal human sera were removed from the sera and from the antigen by adsorption techniques. Seventy per cent of 31 humans with proven psittacosis infections had hemagglutination titer which ranged from 1:10–1:5120. There was poor correlation between the complement fixation and hemagglutination titers.

Complement-Fixation with Beta-propiolactone-Treated St. Louis Encephalitis Antigen. Richard A. Finkelstein and S. Edward Sulkin, The University of Texas Southwestern Medical School, Dallas, Texas.

A method is described for preparing a complement-fixing antigen of SLE virus which is non-infective for mice. It is essentially a "high speed" antigen treated in an early stage in its preparation with Beta-propiolactone, a potent virucidal agent. The BPL-treated antigen is relatively stable on storage and may be lyophilized without significant loss of reactivity. The treated antigen retains the same degree of cross-reaction with other members of the encephalitis group as untreated preparations and previously described antigens. Tests with paired patients' sera have indicated its usefulness as a diagnostic aid. The use of BPL as a virucidal agent in the preparation of noninfectious viral antigens

should constitute a distinct advantage over previously described techniques.

Bronchial Aspiration and Urine Studies in Post Poliomyelitic Patients. W. Fahlberg, R. Jackson, J. Mahoney, D. Packard, and W. Spencer, Baylor University College of Medicine and Southwestern Poliomyelitis Respiratory Center.

The presence or absence of cellular and humoral elements in throat cultures, bronchial aspirations and urine and their interplay with microorganisms in relationship to the general health of the post-poliomyelitic patient was studied. Preliminary investigations showed that a rise in the number of phagocytic and squamous cells indicated infection before the appearance of clinical symptoms.

Throat cultures were essentially normal in both distribution and numbers of microorganisms. Bronchial aspirations however revelaed a high carrier rate of *Staphylococcus aureus*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Urine cultures were uniformly positive with a high percentage of *Escherichia coli*, *Monilia albicans*, streptococci and *Pseudomonas aeruginosa*. The latter two observations are probably related to the fact that the patients studied required the respirators and had a high degree of paralysis.

EGG COUNTS AS ESTIMATES OF INTENSITY OF INFECTION WITH *SCHISTOSOMA HAEMATOBIUM*[a]

J. ALLEN SCOTT

A recent paper (Stimmel and Scott, 1956) reported on the regularity of the output of eggs of *Schistosoma haematobium* in consecutive urine specimens from two cases over time periods of 10 to 54 days. The present report concerns a similar study of a larger number of cases for shorter time periods.

Material and Methods

The subjects were male Egyptian laborers varying in age from 10 to 40 years and with sufficient education to read and write simple Arabic. Each one was hired to work in his local village during the short period in which a preparation laboratory was located there as part of a helminthological survey. They were expected during this period of time to pass each urine specimen in a separate container and record on the container the date and time of passage. The collections were thus not under the direct supervision of the author but were supervised by a trained technician. It is probable that instructions were not followed and that for the most part each series consists of a specimen passed early in the morning of each day and one or more additional specimens passed during working hours. A few specimens were passed in the late evening. In view of the daily cycle of egg output reported by Stimmel and Scott (*loc.cit.*) it would appear that these series would be similar to series of random specimens from each individual. The variability might be expected to average somewhat higher than that of random specimens, since the previous study showed that the early morning specimens usually contained the fewest eggs and specimens passed soon after noon contained the largest number. The volume of the specimens in the present series averaged somewhat lower than would be expected. It may be that only part of a passage was presented in some cases or that the specimens were sometimes passed on demand of the technician in charge. All of these faults would tend to exaggerate the variability. Since the variability did not prove to be much greater than would be predicted, and since no other similar data have been reported, it seems worthwhile to place these data on record. The internal evidence indicates that there was no substitution of specimens but that they all came from the designated individuals.

In the local laboratory each specimen was thoroughly stirred and 50 ml. of urine put into a centrifuge tube. The remainder was measured to obtain the total volume of the specimen. When the volume was less than 50 ml. the

entire amount was placed in a graduated centrifuge tube and the volume recorded. The tubes were centrifuged at slow speed long enough to throw down all the eggs, the supernatant urine was removed, and the sediment spread on a ringed slide and dried according to the method of Barlow (1931). The slides were forwarded to the central laboratory where the eggs were counted by experienced technicians under the direct supervision of the author. To maintain high standards of accuracy, the slides were recounted by a second technician who did not know the previous results. Any cases showing discrepancies were re-examined by the author or the laboratory supervisor.

A coefficient of variation was calculated as a measure of variability between specimens for each individual as follows:

$$CV_{ind.} = \frac{100}{\bar{X}} \sqrt{\frac{S\,x_i^2}{n}}$$

where X is the appropriate value for each specimen from an individual subject, $\bar{X}$ is the mean of X_i, x_i is the deviation of each X from the mean X, n is the number of specimens from the subject, and S indicates summation. These individual coefficients of variation were averaged to obtain an estimate of the relative population variability around the mean of individual subjects as follows:

$$CV_{aver.\,ind.} = \sqrt{\frac{S\left(CV_{ind.}^{\,2} \cdot \frac{n}{n-1}\right)}{N}}$$

where N is the number of subjects.

Presentation of Results

Of the 34 cases studied, 28 presented one or more positive specimens. The results of the examination of these 28 cases are shown in table 1. The cases have been listed in order of the magnitude of the mean eggs per unit volume. The figures for mean eggs per specimen shown in the table were obtained by first calculating the number of eggs in each specimen from the volume of urine and the eggs per unit volume, then averaging the values so obtained. It will be noted that 7 of these cases presented one or more negative specimens. Inclusion of zero values in a short series has the effect of unduly exaggerating the size of the coefficient of variation. We will therefore consider first the 21 cases without negative specimens. The coefficients of variation of eggs per unit volume and of eggs per specimen for these 21 cases obviously do not increase in proportion to the corresponding mean values. Coefficients of correlation between means and co-

TABLE 1

Number of Specimens From Each Individual, Mean Volume of Urine, Mean and Coefficient of Variation of S. Haematobium *Eggs Per Unit Volume and Per Specimen.*

Age	Number* of Specimens	Mean Volume of Urine in ml.	Eggs Per 50 ml. of Urine		Eggs Per Specimen		Age	Number* of Specimens	Mean Volume of Urine in ml.	Eggs Per 50 ml. of Urine		Eggs Per Specimen	
			Mean	CV	Mean	CV				Mean	CV	Mean	CV
35	6 (2)	155	0.8	175	2.2	173	40	11	144	94	60	249	61
24	17 (6)	94	1.4	164	2.7	170	20	6	83	107	53	187	71
23	6 (2)	108	2.5	176	2.0	140	23	6	64	128	22	159	16
35	5 (2)	79	3	133	5	140	45	6 (3)	200	131	221	475	219
21	5	256	7	57	42	81	35	5	126	133	95	320	89
19	5	104	9	67	15	47	16	4	78	207	55	298	56
40	5 (2)	189	12	200	32	175	22	4	71	212	40	270	24
28	7	66	15	60	18	78	25	6	102	248	35	509	37
25	5	56	16	74	15	75	17	18	56	322	73	355	64
35	6 (5)	163	22	150	82	165	26	5	89	340	64	698	103
25	5	112	64	28	144	31	12	6	92	349	77	495	55
18	6	216	75	63	271	106	19	13	116	2,902	88	7,329	110
19	6	59	89	11	20	64	22	15	48	3,931	75	3,074	72
28	11	105	93	76	186	71	11	5	55	4,761	46	4,308	41

* Number of positive specimens shown in parenthesis where different from total number.

efficients of variation for both measures were not significantly different from zero. In other words, the absolute variability of counts is proportional to the mean size of the counts but the relative variability as shown by the coefficients of variations is constant throughout the series. Since the relative variability is constant, averaging the coefficients of variations seems to be a valid procedure. For these 21 cases the average coefficient of variation for any single specimen, calculated as indicated above, was 37 per cent for urine volume, 67 per cent for eggs per unit volume and 75 per cent for eggs per specimen.

We must now consider the effect of adding the 7 cases with some zero counts. The coefficients of variation shown in the table are much larger than those of the other 21 cases, as would be expected. The average coefficient for urine volume based on all specimens from these 7 cases is 43 per cent and is thus not appreciably larger than that for the 21 cases. When a similar figure is calculated for these 7 cases, omitting the negative specimens, the value rises to 51 per cent. This change must be attributed to the very small number of positive specimens. When we consider similar coefficients for eggs per unit volume and eggs per specimen, we find that for the 7 cases including zero counts they are 193 and 186 per cent respectively, and excluding zero counts 113 and 108 per cent respectively. The effect of the zero values becomes obvious, and it is almost equally obvious that, by analogy with the figures for urine volume, we could expect the coefficients calculated without including zeros to be higher than those for the 21 cases because of the small number of positive specimens. When we average the values for all 28 cases including zero counts we obtain coefficients of 39, 113 and 114 per cent.

Discussion

It is hoped that this report will stimulate someone to do a similar study under more rigidly controlled conditions and especially to include longer series of specimens from people whose counts are not consistently positive. Perhaps the procedure should be modified so as to count the entire specimen when the 50 ml. portion is negative. We have not mentioned in this paper the clinical problem of making a positive or negative diagnosis, nor the epidemiological problem of determining the true prevalence

of infection. These problems as well as our present problem of estimating the variability of output are intensified by the fact that certain cases will pass some negative and some positive specimens. The present data add little to the solution of these problems. It would seem, however, that they could all be solved by more extensive studies of this type.

The data presented here, added to the information already available, seem to support the argument that quantitative studies of *S. haematobium* infections are a possibility. As stated by Stimmel and Scott (*loc. cit.*) there are two aspects to the problem. First, can quantitative examination of one specimen give a fair estimate of the average egg production? Second, is the average egg production correlated with the severity of clinical symptoms? Until the first question is answered the second cannot be attacked. The two cases included in the above mentioned study showed coefficients of variation of 27 and 44 per cent for eggs per unit volume and 66 and 80 per cent for eggs per specimen. The minimum figures in the present study are of the same order of magnitude, namely, 67 and 75 per cent for eggs per unit volume and eggs per specimen respectively. It appears that the comparable figures for the entire series, 113 and 114 per cent, are spuriously high because of the presence of negative counts in short series. That the negative counts must be included is obvious since a survey based on a single random specimen from each individual will necessarily include some negative specimens from positive individuals. Until a proper correction factor can be determined from additional studies, it seems sound to estimate that the ultimate values will be somewhat higher than the minimum values and not nearly as high as the maximum values discussed here.

It should be emphasized that these coefficients of variation pertain to individual random specimens, and, while at first sight they appear to be high, they are no higher than similar values for intestinal nematode parasites for which quantitative estimates have been successfully used for decades. As pointed out by Stimmel and Scott (*loc. cit.*) no one experienced with such quantitative methods expects to be able to estimate the intensity of infection of an individual. Interest is centered on accurate estimates of the average intensity in groups of people and for this purpose relatively high individual variability is not crucial.

Summary

Several urine specimens were collected each day for several days from each of 28 young men infected with *Schistosoma haematobium*. The egg content of the specimens was estimated by egg counts and the variability between specimens determined. Quantitative estimates of the average intensity of infection in groups of people seem warranted although more studies of this type are recommended.

REFERENCES AND NOTES

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Stimmel, Carolyn M., and J. Allen Scott: The Regularity of Egg Output of *Schistosoma Haematobium*. *Tex. Repts. on Biol. and Med.*, 14: 440–458, 1956.

[a] Contribution from the Laboratory of Helminth Research, Department of Preventive Medicine and Public Health, The University of Texas Medical Branch, Galveston, Texas. The data were collected under the auspices of the Rockefeller Foundation. Received April 29, 1957.

ELEVENTH ANNUAL SYMPOSIUM ON FUNDAMENTAL CANCER RESEARCH

THE UNIVERSITY OF TEXAS
M. D. ANDERSON HOSPITAL AND TUMOR INSTITUTE
HOUSTON, TEXAS

INTRODUCTION

R. Lee Clark, Jr.*

Fundamental research on the cancer question has, through the years, led to interesting speculation, theorizing and investigation. Perhaps the most current, or "popular" thought on the causation of cancer in recent years, has been the revivification of the theory that viruses play an all-important role in the origin of cancer. For the Eleventh Annual Symposium on Fundamental Cancer Research, we have brought into focus this interest in virus tumorigenesis. In order to accomplish this we sought the council of eminent virologists to advise on this program on "Viruses and Tumor Growth." Our thanks goes to the program committee, Drs. Joseph W. Beard, Duke University; Edwin H. Lennette, California Department of Public Health; Albert B. Sabin, University of Cincinnati; Jerome T. Syverton, University of Minnesota; and the program chairman, Leon L. Dmochowski of M. D. Anderson Hospital, for having arranged to have the outstanding virologists and cancer researchers present this Symposium.

This year, as last, the first day of the Symposium was devoted to annual progress reports on research projects currently under investigation at M. D. Anderson Hospital and Tumor Institute. This year, 34 of the one hundred research projects were reviewed. The research consultant panel offered many helpful suggestions, and we would like to thank the members of the consultant panel: Drs. H. B. Andervont, Murray M. Copeland; Windsor Cutting, Charles D. Kochakian, Theophilus S. Painter, Fred Stweart, and Herman Wigodsky.

The Bertner Foundation Award was presented to Dr. John J. Bittner, for his inspirational research on the various factors involved in the origin of mammary cancer in mice. He theorized and proved that the interaction of three causative factors are involved: the mammary tumor milk agent, inherited susceptibility, and hormonal stimulation. His findings have led to a

* Director and Surgeon-in-Chief, The University of Texas M. D. Anderson Hospital and Tumor Institute.

multitude of other investigations in the field of cancer. The Bertner Award, established in 1950, to honor the first acting director of M. D. Anderson Hospital and the first president of the Texas Medical Center, the late Dr. E. W. Bertner, is conferred annually in the form of a medallion to a person who the Awards Committee feels has made an outstanding contribution to the field of cancer research.

Each year, all papers presented at the Symposium are published in TEXAS REPORTS OF BIOLOGY AND MEDICINE through the courtesy and cooperation of its editor, Dr. John Sinclair, of The University of Texas Medical Branch, Galveston. His splendid cooperation in carrying on this tradition is deeply appreciated.

ABSTRACTS OF 1957 RESEARCH PROGRESS REPORTS

Purification Studies on Follicle-Stimulating Hormone. S. L. Steelman.

Follicle stimulating hormone (FSH) from the pituitaries of swine and sheep has been isolated in highly purified form. Fractionation is accomplished on the anion exchanger, DEAE-cellulose, using gradient elution. The materials so prepared are the most potent FSH preparations yet reported and some show a high degree of homogeneity. They possess distinctive chemical and physical properties and are not appreciably contaminated with other pituitary hormones. As little as 1–2 gamma will increase significantly the ovarian weight in the hypophysectomized rat.

Studies of the Chemistry of Leuteinizing Hormone (LH). Darrell N. Ward.

In a study growing out of our department's interest in the role of the pituitary gland in azo dye carcinogenesis, the purification of LH obtained from sheep glands has been studied. Preliminary purification by a number of published methods indicated the best yields were obtained using the ethanol aqueous actate buffer procedure of Koenig and King. With this as the starting material, or with the Armour LH standard (227–80), the chromatographic behavior has been studied using DEAE-cellulose or CM-cellulose ion exchange resins. Our assay for the hormone has been frog sperm release for a rapid qualitative test, and the rat ventral prostate assay for quantitative evaluation of potency. The best LH preparation obtained by chromatography is now being studied with respect to its purity and chemical properties.

Studies of a New Component in Hormone Feed-Back Mechanisms. J. B. Trunnell.

It is obvious that the feedback systems which maintain homeostasis of hormonal concentrations in body fluids must contain a sensing or sampling device which continuously measures the hormonal level. On the assumption that such centers may be lo-

cated in or near the hypothalamic-pituitary region, serial sections of this area have been made after the injection of isotopically labeled hormones into mice. Autoradiograms of these sections have revealed small nuclei posterior to the hypothalamus and in the cerebellum which concentrate the label following estradiol administration. Other autoradiograms, from animals given labeled progesterone, testosterone, hydocortisone, thyroxine, cholesterol and glucose, are being studied.

Metabolism of C^{14}-Estrone by Azine Treated Rats. Tad L. Patton.

It has been shown in these laboratories that sufficiently large doses of p-hydroxypropiophenone azine will produce the same effects on the accessory sex organs of male rats as beta-estradiol. Also, it was shown that simultaneous dosages of 0.5 microgm. estradiol and 2.0 mgm. of the azine produced the same effect on the accessory sex organs as either one given alone. In order to determine the effect of the azine on estrogen metabolism a comparison is being made of the C^{14} metabolites excreted by control and azine-treated male rats which were injected with C^{14}-estrone. It has already been found that the excretion patterns are different; the separation and identification of the metabolites is now underway.

The Effect of TSH on the Sulfhydryl Groups of the Chick Embryo Thyroid. Beatrice F. Dennis.

The widespread incidence of sulfhydryl groups in connection with such vital metabolic activities as cellular respiration, glycogen and fat metabolism has stimulated the study of the role of sulfhydryl groups in the trapping of iodide by the thyroid gland. Using the chick embryo thyroid, the normal sulfhydryl-disulfide picture has been demonstrated by means of histochemistry. Variations of this pattern have been shown in the glands of B^{12} deficient embryos. Postulating that sulfhydryl enzymes might act as H carriers to activate thyroid proteolytic systems, TSH was given to determine if any alteration in the SS-SH picture occurred.

Role of Folic Acid in Metabolism. V. M. Doctor.

Preliminary studies have been conducted on the role of folic acid in the incorporation of serine-3-C^{14}, formaldehyde-C^{14} and

formate-C^{14} into methyl group of methionine by freshly prepared extracts of chick liver acetone powder. The incubation system consisted of enzyme preparation, one of the three C^{14} compounds listed above, homocysteine, folic acid, $MgCl_2$, ATP and DPN. Omission of any one of the cofactors from the system resulted in a significant reduction in the incorporation of serine-3-C^{14} or formaldehyde-C^{14} into methionine. Formate-C^{14} was not incorporated into methyl group of methionine even in presence of all the above cofactors. Addition of aminopterin to the incubation system gave a reduction in the incorporation of serine-3-C^{14} and formaldehyde-C^{14} into methyl group of methionine. These results have been interpreted to suggest that the reactions leading to the synthesis of methyl group of methionine are mediated by a folic acid derivative.

Melanogenesis in Melanoma and Related Lesions. Arthur J. Speece.

Slides of human melanoma tissue were incubated in tyrosine-2-C^{14} and sectioned from paraffin. The paraffin sections and radioautographs prepared from them, were measured for optical density in corresponding microscopic fields by the use of a microspectrophotometer. The sections were then stained so that the measured areas could be evaluated for cytopathologic change. Studies by this method on melanoma and other pigmented lesions have indicated that melanin production is most rapid in fast-growing tumor cells. Melanin is transported from tumor cells to extracellular disposal areas at rates which vary among patients and are independent of rate of production.

Purification of Mammalian Tyrosinase. F. Christine Brown.

Tyrosinase from plant sources has been highly purified and characterized to some degree. The enzyme from mammalian sources has been reported to be associated with cellular particulates in such fashion that it cannot be purified by conventional techniques. A product has been obtained from mouse melanoma by acetone-salt fractionation which has at least ten times the activity of the starting material and with 20 per cent of the original

activity. The product is a white powder, ruling out the possibility of tyrosinase being associated with melanoprotein. Chromatography on DEAE-cellulose columns resulted in an additional 30-fold purification, assuming no loss of activity during the procedure. Preliminary experiments indicate that tyrosinase is stable to tryptic digestion.

Histochemical Study of Early Changes in Rat Liver During 3′-Methyl-4-Dimethyl-Aminoazobenzene Carcinogenesis. J. P. Chang.

After two weeks of feeding male Sprague-Dawley rats with 3′-methyl-4-dimethyl-aminoazobenzene, remarkable changes in alkaline phosphatase, esterase, mitochondria, glycogen and ribose nucleic acid were found in the liver. Changes in succinic dehydrogenase, acid phosphatase and lipid were not significant. These early changes, which occurred before bile duct proliferation and before any appreciable morphologic alterations, may well be of prime importance in liver carcinogenesis.

Influence of Radiothyroidectomy Upon the Induction of Leukemia in DBA Mice by Methylcholanthrene. Noriaki Ida.

Although 75 per cent (16/21) of Dba/2 male mice developed leukemia when skin-painted 18 times in 6 weeks with 0.25 per cent solution of methylcholanthrene in benzene, only 13 per cent (5 of 39) of the radiothyroidectomized males became leukemic when similarly treated. If DBA male mice were both castrated and radiothyroidectomized, 39 per cent developed leukemia. On the basis of survival time and weight, it is apparent that the effect of radiothyroidectomy is real and that it is modified by castration.

The Potentiating Effect of Urethane on the Induction of Mouse Leukemia by X-Rays. Sadahisa Kawamoto.

C57 BL mice received whole body radiation with 440 r x-rays given in fractional doses, 40 r every 4 days for 11 times. A second group received the same treatment with x-rays, but with

the thigh shielded during irradiation under urethane anesthesia. There were 52 mice in each group. Of those receiving whole body x-radiation, only four developed leukemia. In the group receiving urethane in addition to x-rays, 49 became leukemic. Although mice of the latter group were thigh-shielded during irradiation, they were not protected against the induction of leukemia, apparently because of the administration of urethane which potentiated the leukemogenic action of x-rays.

Transketolase-Transaldolse Pathway of Pentose Synthesis in Lymphatic Tissues and Tumors. Saul Kit.

Normal and malignant lymphatic tissues were incubated with glucose-1-C^{14}, glucose-2-C^{14}, or glucose-6-C^{14} and the labeling of the pentose moiety of the ribonucleic acid-purine nucleotides was measured. Approximately twice as much radioactivity was converted to pentose when glucose-2-C^{14} was used as substrate as when glucose-6-C^{14} was the substrate. Also, more radioactivity from glucose-1-C^{14} was converted to ribose than from glucose-6C^{14}. In some instances, the ribose was degraded and the radioactivity of carbon five determined. The results suggest that approximately half of the labelled RNA-ribose was derived via the transketolase-transaldolase pathway and half via the direct oxidative pathway.

Action of Nitrogen Mustard (HN_2) on Thymine Metabolism in Patients with Leukemia. J. Awapara and C. C. Shullenberger.

After the administration of nitrogen mustard, patients excrete large amounts of β-aminoisobutyric acid. This amino acid is formed from thymine. This conversion has been demonstrated in patients. The mechanism by which thymine is produced after administration of the mustard is under investigation. Quantitative studies thus far indicate that the greatest excretion of β-aminoisobutyric acid occurs in patients with lymphosarcoma or chronic lymphocytic leukemia. Patients with Hodgkin's disease or bronchogenic carcinoma excrete much less.

The Pathogenesis of the Anemia of Hodgkin's Disease, Lymphosarcoma and Chronic Lymphocytic Leukemia. Daniel E. Bergsagel.

Erythrocyte survival (using Cr^{51}) and ferrokinetic (using Fe^{59} citrate) studies on normal and anemic patients with chronic lymphocytic leukemia, lymphosarcoma and Hodgkin's disease for a quantitative analysis of the mechanism of the anemia. Preliminary studies have revealed a marked difference in the erythropoietic activity of the marrow in chronic lymphocytic leukemia and Hodgkin's disease. In chronic lymphocytic leukemia erythropoiesis was reduced with low reticulocyte counts and reduced plasma iron clearance rates (normal or elevated plasma iron, prolonged $T\frac{1}{2}$ Fe^{59}); in Hodgkin's disease erythropoietic activity was increased with reticulocytosis and increased plasma iron clearance rates (low serum iron but greatly shortened $T\frac{1}{2}$ Fe^{59}). The reduced erythropoiesis in chronic lymphocytic leukemia might be due to reduced stimulation or inability of the marrow to respond to adequate stimulation. Therefore the erythropoietic stimulating factor will be assayed in anemic plasma from patients with each of the three diseases and compared with the activity of normal plasma. The uptake of Fe^{59} by erythrocytes in hypophysectomized rats will be used to assay this factor.

Intracranial Complications in Children with Acute Leukemia. Margaret P. Sullivan.

Involvement of the meninges and brain parenchyma in untreated patients with leukemia is not unusual and occurs in acute and chronic disease regardless of cell type. Children with leukemia followed at this hospital in the last 16 months have developed signs of this complication. The pathogenesis of this syndrome is unknown. It appears that such agents as 6-mercaptopurine and A-methopterin cross the blood-brain barrier in greatly reduced amounts and fail to control the intracranial progression of leukemia even though the disease may be under fair control elsewhere in the body. The most effective treatment for this complication at the present time is x-ray therapy to the entire skull.

Microbiological Studies on Derivatives of 4-Aminopyrazolo (3,4-d)pyrimidine (4APP) and Other Antimetabolites. Robert Fuerst.

An adenine analogue, 4-aminopyrazolo(3,4-d)pyrimidine (4APP) has been shown to be a potential chemotherapeutic agent against transplantable mouse tumors. Attempts were made to determine its effect on the metabolism of strains of *Neurospora crassa.* Structure-activity relationships of fifty-seven 4-APP derivatives were studied. Nineteen of these compounds inhibited growth of *Neurospora* in direct relation to their chemical structure. The positions of side groups on the molecules determine the interaction between inhibition and relief potential of these groups, depending upon molecular concentration. Three types of inhibition curves were found to be related to the chemical structure of the active 4-APP derivatives. Twelve 4-APP derivatives and sixteen other compounds related to pyrimidine gave relief of 4-APP inhibition. Relief data suggests two possible potential pathways of 4-APP action in *Neurospora*—those of thiamine synthesis and DNA synthesis.

Developmental Pathology of Bovine Ocular Squamous Carcinoma. E. Staten Wynne.

Of 533 Hereford cows over 5 years old, 12 per cent had carcinoma and 64 per cent benign percursor lesions. The accuracy of clinical diagnosis was 78 per cent. Sixty-four per cent of the lesions arose in the conjunctival sac, with 35 per cent on the lateral limbus. There was a highly significant trend toward bilaterality and multiplicity. Complete regression occurred in about 14 per cent of all lesions, including 4 per cent of carcinomas. Seventeen per cent of operated lesions recurred. Pigmentation of lids, nictitating membrane, and limbus may be of paramount importance in protecting against this disease.

A Biological, Biochemical and Biophysical Approach to the Study of Mammary Cancer in Mice. Leon Dmochowski.

In a series of extensive biological tests the milk of mice carrying the mammary tumor-inducing virus has been shown to be

the best source of the virus. Study of the distribution of the virus in various fractions of milk has shown the virus to be present in the highest concentration in the microsomal fraction of milk. This fraction contains particles of approximately 300 Å diameter. Similar size, active particles have been found by ultrafiltration through collodion membranes and by differential ultracentrifugation of various preparations of the virus. Enzymes such as prostate phosphatase and snake venom diesterase do not affect the activity of virus preparations. Electrophoretic analysis, so far, has shown no difference between the milk of virus-carrying and virus-free strain mice. Comparison of the results of bioassays of tumors studied in the electron microscope with electron microscope findings of these tumors suggests, but does not prove conclusively, that characteristic virus particles seen in mammary tumors are the specific virus. The size of the active particle is similar to that of the internal dense zone of the virus particles seen in the electron microscope. The virus does not induce any visible changes in the developing chick embryo or in mouse embryo cells and HeLa cells grown *in vitro*.

C^{14}-Infected Estrogen Metabolism in Mice Infected with the Mammary Cancer Agent. W. E. Bowman.

The distribution of carbon labeled estrogen in strain A mice is being studied. The test animals are those infected with the milk-transmitted agent while foster-nursed mice of the same strain serve as controls. The investigators are not informed as to the identity of these animals until the conclusion of the experiment. The mice are kept in glass metabolism cages for twenty-four hours after the injection of the labeled estrogen and are then sacrificed. Estrogen extracts are made from urine, feces and liver and are chromatographed on Celite columns. Crude extracts from other organs are also prepared. All extracts are plancheted and counted for radioactivity. Data from three such experiments indicate that the virus-infected mice may possess estrogen metabolism and excretion patterns which differ from those of the controls.

Influence of Ovariectomy and Adrenalectomy on the Growth Rate of Spontaneous Mammary Carcinoma in RIII Mice. Arthur Kirschbaum and Nylene E. Eckles.

Mammary carcinoma occurs spontaneously in RIII virgin and breeder female mice. In virgins age of appearance of most cancers is 10–12, in breeders 5–8 months. Absolute weight of mammary cancer is obtained in living mice by appropriate calculations. Rate of growth is more rapid in young (breeder) than old (virgin) mice. In the former ovariectomy depresses, in the latter accelerates growth rate. Adrenalectomy suppresses in both groups. Response of RIII mammary cancer to hormonal therapy simulates response of human metastatic breast carcinoma, suggesting investigative possibilities. Effects of adrenalectomy *per se*, pituitary isografts and exogenous sex steroids are being studied. Lactogenic effects of pituitary isografts are being associated with inhibition of tumor growth. Prolonged lactation is also suppressing tumor growth in RIII mice.

Physical and Biological Studies of the Radiation Fields Around Interstitial Needles Loaded with Various Radioactive Sources. Jane Blizard.

The influence of sheath design around interstitial sources of radium, Cobalt-60, Cesium-137, Gold-198, and radon has been studied by calculation, photographic film, scintillation counter, ion chamber and biological techniques. Commercial Cobalt-60 needles have a stainless steel sheath of smaller diameter than standard radium needles. Cobalt-60 needles have been found clinically to give an abnormally high dose rate close to the needle surface. Both the physical techniques and the biological measurements on rabbit skin confirm the effect. The influence of homogeneous and non-homogeneous distribution of activity in interstitial needles has been investigated. The clinical significance of these effects will be presented.

The Relative Biological Effectiveness of 200 KV X-Rays, Cobalt-60 Gamma-Rays and 22 MV X-Rays. W. K. Sinclair.

Determinations of the RBE of these three radiations have been made using defined dosimetry conditions. The biological tests so

far include LD-50 in mice, LD-50 in rats, and the uptake of radioactive iron in the red blood cells of rats. Results so far show RBE's of the order of 0.8 for the two supervoltage radiations relative to 200 KV.

The Properties and Relative Biological Effectiveness of Iron-55 and Iron-59. Arthur Cole.

Radiation killing of bacterial spores by Fe-55 and Fe-59 was studied quantitatively. Quantitization presupposes a knowledge of the radiation properties. The properties measured for Fe-55 were: 1) Auger electron range = 100 ugm/cm^2; 2) Auger electron true backscattering = 5 per cent; 3) K fluroescence yield = 0.29 $\pm$ 0.015. Radioactive deposits on foils could be standardized to better than 5 per cent accuracy for both Fe-55 and Fe-59. The accuracy of secondary standardization of Fe-55 solutions was $\pm$ 15 per cent. Biological tests were made using survival of *Streptomyces griseus* spores, suspended in solutions of the radioisotopes, as an indicator. A relative biological effectiveness of Fe-55 compared with Fe-59 of 1.07 $\pm$ 0.245 was observed for this system.

The Compartive Effects of Cobalt-60 Gamma Rays and 200 KV X-Rays on Solutions of Lysozyme. R. J. Shalek.

Solutions of lysozyme of concentration of 15 μg/ml or less show partial inactivation with radiation doses of 100 to 500 rads (approximately 100 to 500 roentgens exposure). For the same inactivation about 20 per cent more energy absorbed is required for x-rays than for Cobalt-60 gamma rays. Both Cobalt-60 and x-ray irradiations show the same variation of sensitivity with pH and temperature, suggesting that pH change or heat in the particle track are not important in the action of the radiation.

Tumor Localization Techniques Using Radioisotopes. Raymond G. Rose.

Various radioactive isotopes and labelled substances are being applied to the problem of detection and localization of certain tumors which are difficult or impossible to detect by means of standard diagnostic procedures. The most successful such appli-

cation has been that of radioactive iodine in thyroid cancer. Useful also is the diagnosing of possible intraocular tumors with radioactive phosphorus and of brain tumors with radioactive serum albumin. More recently, studies have been undertaken at this institution to delineate the outlines and the functional and architectural integrity of organs such as the liver and pancreas.

Tissue Culture Studies on the Thyroid Gland. George G. Rose.

The first block of work was an evaluation of the effect of chicken embryonic and whole egg juices and ultrafiltrates on the 8-9 day chicken embryonic thyroid *in vitro*. The work thus far carried out on 400 thyroid explants indicated that all three markedly stimulated the epithelial elements but not the stromal elements. The second block investigated concurrently involved time-lapse studies on the secretory ability of the embryonic epithelium with an interference-color-phase-contrast microscope. Follow-up studies with I^{131} and autoradiography indicated the secretory phenomenon observed as potential thyroid activity of the emigrating epithelium.

Heteroploidy in Human Cell Strains. T. C. Hsu.

Eleven human cell strains, six neoplasms (HeLa, S-3, KB, Oregon J-96, Oregon J-111 and Maben) and five strains derived from nonmalignant tissues (Chang's Liver, Conjunctiva, Henle's Intestine, and Pomerat's McCoy and FF) were proven to be heteroploids. None of the so-called "normal" strains was normal, as far as the chromosomal constitution is concerned. A new fibroblast strain, known as Mayes, was studied to follow the chromosomal patterns in every transfer generation. The primary culture and the first few generations showed extensive diploids. The transformation occurred in the sixth and seventh generations. In the eighth generation, all the cells were heteroploids.

Anomalies in the Optical Rotatory Dispersion of Serum Albumin Isolated from the Blood of Cancer Patients. B. Jirgensons.

The optical rotatory dispersion of 28 highly purified human serum albumin specimens was studied. All of the albumin specimens which were isolated from normal blood or from individuals

with non-severe, non-malignant ailments showed normal rotatory dispersion. Only five of the 20 albumins which were from individuals suffering from malignant diseases had the same dispersion constants as normal albumins. The other fifteen samples exhibited slight abnormalities which consist chiefly in that the cancerous albumins have slightly lower dispersion constants than the normal albumins.

Transthoracic Needle Biopsy of Parietal Pleura in the Differential Diagnosis of Pleural Effusion. Melvin Samuels.

During the past year transthoracic parietal pleural biopsy with Vim Silverman needle was done in fifty-three cases, forty-seven of which were malignant pleural effusions. The site chosen for biopsy routinely was the ninth or tenth interspace in the area of the mid scapular line. There are four categories of study as follows: bronchogenic carcinoma, breast carcinoma, malignant lymphoma, and a miscellaneous group. 1) Bronchogenic Carcinoma: 18 cases; 12 positives, 3 reaction fibrosis, 10 fluid positives, 3 positive biopsy but negative fluid, 2 positive fluid but negative biopsy. 2) Breast Carcinoma: 14 cases; 8 positive biopsy, 2 atypical fibrosis, 7 fluid positives, 5 fluid negative but biopsy positive, 4 fluid positive but negative biopsy. 3) Malignant Lymphoma: 13 cases; 4 positive biopsy, 2 atypical fibrosis, in no case was a cytological diagnosis of lymphoma definitely rendered from fluid study. 4) Miscellaneous: 8 cases: 1-carcinoma of thyroid with positive biopsy, 1-dysgerminoma with negative biopsy and negative fluid, 6-cases of miscellaneous non-malignant conditions were completed including 1 serositis, 1 pulmonary infarction, 1 pulmonary pneumonia, 1 tuberculosis, 2 congestive heart failures. The tuberculosis biopsy was positive which made possible a diagnosis of miliary tuberculosis in 24 hours.

Total malignancies 47. Positive biopsy 25. Positive fluid cytology 17. There were 7 cases of atypical fibrosis and it is our feeling that such a finding is suggestive of malignancy and should encourage additional diagnostic procedures.

The Investigation of the Properties of Ethyl Vinyl Ether. F. A. Carpenter.

Ethyl vinyl ether is an asymmetrical, unsaturated ether resembling both divinyl ether and diethyl ether in chemical and

physical properties, as well as in structure and clinical use. The potency has been judged to be almost twice that of ethyl ether, but not so potent as divinyl ether. Toxicity is shown to be far less than divinyl ether. Ethyl vinyl ether is compatible with all other agents and soda lime. Standard safety tests show that ethyl vinyl ether is as safe as diethyl ether and much safer than divinyl ether. Since 1947, when the first report of a case utilizing a human volunteer was made by Krantz, ethyl vinyl ether has been utilized with all types of surgery and anesthetic techniques.

The Investigation of New Carbon Dioxide Absorbents for Use in Anesthesia for Cancer Surgery. Richard C. Smart.

The efficient removal of carbon dioxide from closed system anesthesia circuits has presented a major problem to anesthesiologists. Soda lime has been used for this purpose for over 25 years, in spite of the fact that its inadequacies have been well documented. This study is an effort to look into the properties of other carbon dioxide absorbents and adapt them for use in anesthesia for extensive surgery.

Chemical Rhizotomy for Control of Pain Associated with Advanced Cancer. Richard C. Hay.

The control of intractable pain associated with cancer is frequently difficult. The narcotics have not proved uniformly suitable. The purpose of this study is to present data gathered from the treatment of a group of patients who have been blocked by various long-acting agents. These data are to be correlated with the pathologic findings from the area blocked. The advantages of this technique over narcotics are discussed.

Personal Counseling as Adjunct to Medical Therapy with Cancer Patients. Alan Krasnoff.

Hypotheses: 1) Patients with personal counseling as adjunct to medical therapy for cancer of the uterine cervix tend to show significant shifts in responses on pre-test, post-test, and follow-up administrations of psychological instruments when compared with patients who had medical therapy alone. 2) Patients having a lesser amount of psychological disturbance tend to have less severe side effect responses to radiation treatment compared to

patients who are more maladjusted. Due to unreliability of certain data, the second hypothesis was modified to test the relationship between side effects and psychological adjustment. Results of the investigation tend to support both hypotheses in part.

End Results of a County-Wide Follow-up Study of Cancer. Eleanor J. Macdonald.

The end results of cancer by anatomic site for a 12-year period will be shown for a Texas county. Included are all patients known to hospitals, laboratories and private physicians' offices. Nearly half the population is Latin American, and will be reported in comparison with the other American group. Incidence and survival rates adjusted for age and sex will be given of the residents of the county. Survival rates will be reported also using the maximum likelihood method for both residents and the 6,000 patients from other states and countries treated in the county and traced.

INTRODUCTION TO VIRUSES AND TUMOR GROWTH

R. Lee Clark, Jr.*

We know that malignant growth is caused by the interaction of a number of factors. Some of these factors are known and others still remain to be discovered. Cancer as a disease often is the end product of a sequential development involving numerous factors, each factor forming a link in the etiological chain. A number of these links are known, such as the genetic, hormonal, and metabolic factors. Viruses are now recognized as having some part in the generation of certain tumors and have been shown to have actual tumor-inducing properties. There appears to be no difference in the various properties of viruses which we know as ordinary viruses and those of the tumor-inducing viruses, except that the former will never induce a continuous unrestricted proliferation of tissues they infect. The facts are uncertain even on this point, as there are reports of the possible involvement of some of the ordinary viruses in at least one type of cancer. These reports, if substantiated, would mean much in the better understanding of the cancer problem.

It is just over half a century since the first report on viruses appeared and was substantiated. Since then the discovery of a new virus has followed almost yearly. Twenty-two years ago, Dr. Stanley published his first paper on the isolation, purification and crystallization of tobacco mosaic virus which led to the first chemical identification of a virus. This gave a new impetus to virus research, and mammalian viruses have since been obtained in equally pure form, progress which even a few years ago appeared almost impossible.

Within the last few years new techniques have successfully been applied to the studies on viruses, leading to a gradual understanding of their characteristics. We now speak not only of the anatomy of viruses but even of histology and growth patterns of viruses which have not only the properties of molecules but also the property to reproduce and mutate. In the chemical sense we

* Director and Surgeon-in-Chief, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

are now able to analyze and dissect them and gain knowledge of their most intimate appearance and character.

These advances in the field of ordinary viruses have contributed greatly to our knowledge of tumor-inducing viruses. There appears to be no doubt that a meeting of virologists and tumor-virologists can be mutually profitable and further the progress of virus research in general and cancer research in particular.

In the discussion of the origin of any disease, and that of virus-induced tumors especially, not only the agent or agents causing the disease should be discussed, but also the affected host. An integrated study of the agent and of the environment in which it acts include factors in the host which either hinder or facilitate the action of the agent, and a study of the ways and means of its spread will lead to a better understanding of the agent and of the disease itself.

This Symposium devoted to a discussion of tumor-inducing viruses, includes many facets of the problem and should further our knowledge of the problem of cancer. Our thanks goes to the program committee, Drs. Joseph W. Beard, Duke University; Edwin H. Lennette, California Department of Public Health; Albert B. Sabin, University of Cincinnati; Jerome T. Syverton, University of Minnesota; and the program chairman, Leon L. Dmochowski of M. D. Anderson Hospital, for having arranged to have the outstanding virologists and cancer researchers present this Symposium.

THE PATHOLOGY OF VIRAL NEOPLASIA

ERNEST W. GOODPASTURE, M.D.*

In the title of this discussion I have used the word neoplasia to indicate a proliferative process which includes a mode of host-cell response to viral infection. Neoplasia in the sense in which I use it is a process which though including tumorous growth is broad enough to encompass also the formation of proliferative lesions which are not tumors as most pathologists define the term; a neoplasia in other words that is not necessarily restricted by a specific connotation of cell autonomy. Viral hyperplasia would be an acceptable alternate term in most instances.

Although a few remarks about meanings which I shall attach to the words neoplasia and tumor might be helpful, for present purposes at least, I assure you I have no intention of trying to define the qualifying word virus. It would be well, however, if the pathologist could define both terms, lest the science of virology appropriating oncological terminology, in exploiting the hypothesis of viral etiology of cancer, unwarrantedly gain support for its thesis by semantic inference rather than by scientific evidence.

Up to the present time virus research does not seem to me to necessitate a basic change in the pathologist's concept of tumor, for that concept can still serve as a useful point of reference for theoretical evaluation of new evidence. Although there are those who are convinced that an attempt to define tumor is a hopeless task, the majority of pathologists I believe would agree with Ewing that a tumor is an autonomous new growth of tissue (Ewing, 1940). This would not mean, according to Oertel, 1927, that tumor cells have acquired a specific property from outside which causes unrestrained proliferation, but rather that there has been a fundamental alteration in intrinsic cellular constitution and metabolic processes which is irreversibly expressed by extraordinary growth behavior. What the change is has not been acceptably determined morphologically or biochemically despite

* Scientific Director, Department of Pathology, Armed Forces Institute of Pathology, Walter Reed Army Medical Center, Washington, D. C.

heroic efforts, but it might amount to a somatic mutation. Willis' elaboration of Ewing's definition is a little more qualified (Willis, 1953). He holds a tumor to be "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change."

With Willis' definition in mind, one can better appraise the implications of several hypotheses involving virus-cell relationship in the origin of cancer, such as the two suggested by Bourne (1952) in his recent book *Cytology and Cell Physiology*. The possibilities which he proposes are: First, virus (presumably from an extraneous source) alters a normal cell in some way so as to induce a kind of mutation of it, and then merely continues to proliferate as a passive passenger (or, as one might add, disappears altogether). In this hypothetical role, virus entering from without would eventually affect cells in the same way as chemical and physical carcinogens do to provoke a state of autonomy respecting reproduction. This state of autonomy would be encompassed by the pathologist's definition of a tumor and its malignant state, cancer. Virus in this sense would be only a remote or provocative tumorogen. Bourne's second hypothesis is that virus (again assuming an extraneous origin) becomes intimately linked with the reproductive apparatus of its host-cell so as to form some sort of irreversible regenerating cell-virus complex which is the immediate or activating cause of tumor and cancer. This would be a permanent change in the reproductive mechanism of the cell, but inasmuch as it would not be exclusively a change of the intrinsic constituents of the cellular reproductive mechanism, if the virus were an invader, it would be something more than a somatic mutation, something which might be a sort of transduction (Luria, 1953).

Such a self-reproducing combination of an originally extrinsic virus and an intrinsic cellular reproductive apparatus might be considered to be the universal tumorogenic infectious agent which some virologists envision, but only so if one would postulate the existence of an original infecting virus which would be ubiquitous as an invader and resident of all cells capable of developing tumors and becoming cancerous. This more remote and universal virus infestation would be in addition to and anteceding any subsequent specific infection or chemical or physical

agency which provoked its ultimate cancerous effects. This assumption might seem to some virologists to be a little cumbersome and entirely unnecessary in view of a third possibility which might explain the virus-cell-cancer relationship by the more simple assumption of a permanent, irreversible autogenous and self-regenerating change in an essential element of a cell's inherent reproductive apparatus, such as a gene, which might be brought about by a number of physical, chemical and biological provocative agents including some infectious agents, and which in its own right might be christened a virus. Inasmuch as every cell capable of becoming cancerous could undergo this intrinsic change, it would not be necessary to assume the previous presence of an ubiquitous virus. It would only be required that the permanently altered and reproducing elements potential in every proliferative cell be called a virus, a cancer virus, and that would explain cancerous somatic mutation in terms of the virus theory. This interpretation would bring us very close to Boycott's criticism, as reported by Duran-Reynals (1950) that, "When it becomes necessary to postulate a normal virus occurring in normal cells one had better call it something else than a virus." I fully agree and suggest that the spontaneous generation in the cell of a self-perpetuating cancerogenic agent of this sort should better be called a mutant-gene rather than a virus, at least for the time being.

Although any one of the three situations which I have mentioned might be proposed to hypothecize an etiologic relation of virus to tumors, there is a fourth and to me preferable explanation of virus neoplasia which does not require the assumption that so-called virus neoplasias are autonomous growths. It does not appear to me to be justifiable at present to assume that virus has anything more to do with the tumor-cancer problem than do chemical and physical carcinogens which affect the cell in such a way as to increase the probability of an irreversible, permanently reproducible, presumably non-viral change in its intrinsic reproductive apparatus, which when once established continues on its own, independent of further provocation. This remote effect might be a somatic mutation which would be encompassed by the pathologist's present definition of tumor as an autonomous new growth. But until and unless this intrinsic change should occur in proliferating cells initially infected with virus, and be

demonstrable in the absence of virus, the new growth stimulated by the presence of an indwelling and replicating virus would not properly be called tumor nor cancer even though the growth exert malignant effects. It would better be called a viral hyperplasia or neoplasia under the assumption that should virus disappear or cease to irritate the infected cell, growth would regress and the hyperplastic tissue would become quiescent or be absorbed, because the essential reproductive mechanism would be unimpaired.

It would seem reasonable to me to assume that all new growths associated with virus infection are non-tumorous, viral hyperplasias until an autonomous cancerous activity should be demonstrated in the affected cells, perhaps by tissue culture, grafting or otherwise, in the absence of virus. Even though virus should persist in proliferating cancerous cells, it would not be contrary to experience to consider it to be a passive passenger.

It is from this point of view that I should like to discuss a few well-known examples of new growths which to me are explicable as viral hyperplasias rather than as viral tumors if the latter term suggests anything more than an indirect or provocative cancerogenic change in the affected cells.

I should like to present the illustrative infections in three categories:

Group I. Virus infections presenting lesions of self-limited growth. This group can be illustrated by members of the pox group: Fowlpox, molluscum contagiosum, the rabbit myxoma-fibroma complex and by most warts.

Group II. Virus-initiated neoplasia presenting lesions of both limited and unlimited growth but in which evidence of the presence of virus eventually may disappear from the latter. This group may be illustrated by cutaneous warts of rabbits, some of which eventuate in carcinomas. This is the only well established example of this category, where virus is remotely provocative of cancerous change.

Group III. Virus-initiated neoplasia represented by lesions, often of unlimited growth, in which evidence indicates a persistence of virus possibly as a continuous growth stimulating agent, or as a passive passenger, even though at times it may not be detectable, that is, be masked. This category may be illustrated by the so-called Rous sarcoma of fowls.

There are many other proliferative lesions in which virus is suspect or proved to be of etiological significance, such as adenohyperplasia and carcinoma of the breast of mice in relation to the so-called milk factor, leukemia of mice, Lucké's renal tumor of frogs and some growth in plants which, no doubt, fall within one of these categories. There are no examples at present of so-called virus tumors in man to be included, other than warts and molluscum contagiosum which are sometimes referred to as tumors.

To begin with, let me illustrate the first group of self-limiting virus neoplasias by showing you some effects upon proliferation of cells in one of the oldest known virus diseases, one so characterized by proliferative lesions that Borrel, the first investigator to envision a virus-cancer causal relationship, named it epithelioma contagiosum. This is an infection of birds, especially of domestic poultry, known as fowlpox.

The virus of fowlpox under field conditions has a considerable propensity for stimulating infected epithelial cells of the skin and crop to multiply so that hyperplastic nodules appear particularly about the head in unfeathered areas and on the legs. It also brings about infections of squamous epithelia of the mouth, nose and crop which ulcerate, become infected by extraneous microorganisms and thus not uncommonly lead to death. In survivors immunity supervenes after several days and the lesions disappear, although detectable virus may persist in the blood and organs for several weeks or even months.

In a long experience with this disease under experimental conditions in the laboratory, I have never observed a lesion of fowlpox become persistent or cancerous, notwithstanding the implication of fowlpox virus by Duran-Reynals in the development of cancer of the skin of chickens following the application of methylcholanthrene (Duran-Reynals, 1952).

I have been able, however, to observe under experimental conditions mutants of a field strain of fowlpox virus which have a greatly increased potency for stimulating certain cells to proliferate, and possess the ability to affect types of cells not demonstrably altered by the parent strain, such as, for example, renal epithelium and osteoblasts. These mutations may be brought about or be selected by injection of suitable quantities of virus of field strains into tissues in which no lesion occurs in the na-

tural disease, such as brain, bone and subcutaneous tissue of baby chicks; and then reculturing on the chorio-allantois of chick embryos. After one or more such passages alterations in the membranal lesions appear which indicate the presence of mutants, notably the dissociation of epithelial cells of the chorio-allantois, pseudopodial activity of the cytoplasm of the "free" cells and certain morphological changes which are at variance with those characteristically resulting in the pathognomonic Bollinger body of the classical lesion.

Such a mutant strain injected into the kidney of 2–4 day old chicks led, over a period of several inoculations, to remarkable focal proliferative changes in renal cortical epithelium and subsequently in the same animals to proliferative involvement of the endosteum and periosteum of bones of the extremities in focal areas especially about the ankles; but sometimes involving also the wing bones.

The infection with these mutant strains is practically always lethal in young chicks, but in cases of recovery evidence indicates that the proliferative lesions succumb to necrosis and resolve with healing. These chicks on recovery are immune to subsequent infection with fowlpox.

Some of these lesions for a while superficially resemble tumors, but it is evident they are really self-limited, virus-induced neoplasia or, as I would rather consider them, viral hyperplasias.

In human beings the papules of molluscum contagiosum, sometimes referred to as tumors, and the viral warts called papillomas commonly occur in young persons and resolve with development of immunity. Rare instances of carcinoma developing at the site of condylomata have been recorded. Among animals numerous examples of proliferative lesions due to specific viruses might be cited which resolve with the development of immunity. Ectromelia of mice, myxomatosis and fibroma of rabbits as well as numerous instances of species specific warts are well known examples. In these often highly proliferative viral lesions there is no evidence of irreversible change in the cellular growth mechanism because with the onset of immunity growth ceases.

Although most warts of man and animals are self-limited growths, undoubted carcinomas have been observed to arise with great frequency under experimental conditions in one variety occurring in rabbits. This fact afforded proponents of the theory

of virus etiology of tumor strong encouragement for a while. The type of wart referred to is the well-known Shope papilloma of cottontail rabbits (Shope, 1933), which may eventuate in cancer in the natural host, but does so somewhat more commonly when the infection is established in the skin of domestic rabbits. This growth illustrates my second category of virus neoplasias in which virus acts as provocative of the cancerous change.

Syverton and his collaborators were unable to demonstrate virus in cancers that developed from warts in wild cottontails and could not detect antiviral antibodies in rabbits into which cancerous tissue was grafted. Nor did cancerous tissue from Western cottontails induce papillomas in the susceptible Eastern variety. Syverton (1952) therefore concluded that results of their immunological study supported the hypothesis that the role of papilloma virus in the rabbit papilloma-to-carcinoma sequence is limited to that of a *provocative* carcinogenic agent and is not an essential component of the cancerous transformation.

Kidd (1950) likewise came to this conclusion after studying succesive generations of his V_2 strain of carcinoma which in time lost every evidence of associated papilloma virus. It seemed clear to him on the evidence that papilloma virus is not necessary for continuing growth and malignancy of this tumor, and he therefore came to regard the virus as of the category of a carcinogenic agent such as tar, arsenic, methylcholanthrene and many other substances which bring about a change in tissues so that cancers arise in them but otherwise play no part in continuing the malignancy.

In my third category of virus-induced neoplasias, illustrated by Rous sarcoma No. 1 (Rous, 1936), there is evidence of persistence of virus or its constituents in a type of growth which has in its usual manifestations many formal similarities to cancer.

This is the type which offers the greatest difficulty to interpretation of the relation of virus to "tumor" and "cancer." It does not exactly fit the first group of proliferative lesions which characteristically demonstrate their lack of autonomy by cessation of growth and resolution with the development of immunity, although Rous neoplasms not infrequently become quiescent and even completely regress. The Rous sarcoma virus may even induce a self-limited disease, but apropos this discussion, it causes a growth which invades and metastasizes to result in death.

Virus may be readily demonstrable or become masked in this neoplasm, though it probably never disappears completely as it seems to do in carcinomas derived from virus papillomas of rabbits. The question is, does it act as a constant irritant without altering essentially the cell's reproductive mechanism, so that with its disappearance cessation of growth would result and resumption of an orderly physiological state would ensue; or does the virus cause an irreversible change in reproductive mechanisms of the infected cells, which thereby become cancerous, although virus continues to persist simply as a passive passenger.

Rous sarcoma No. 1 virus can behave much like a self-limited infection as was shown by Duran-Reynals (1940–41) in baby chicks in which infection induces not neoplasia, but hemorrhagic lesions probably associated with vascular necrosis. In old fowls, furthermore, the proliferative lesions seem to subside and are held in control. But most typically this virus induces growth resembling sarcoma, growing, invading, metastasizing and killing. It is a question though whether metastases might not sometime be viral in origin rather than resultant from cellular deportation and localization as in true cancer. Virus for instance is more resistant to the inactivating effect of radiation than is the host cell, and Peacock (1946) was able, by irradiating a Rous sarcoma growing in a fowl's wing, to observe within a month multiple growths arising about the periphery of the inactive or destroyed nodule. These satellite growths were not due to cells invading from the irradiated lesion nor were they necessarily due to locally liberated virus either, apparently, for irradiation of similar non-viral implanted fowl sarcomas did not develop satellites, but if Rous No. 1 virus were injected into a breast muscle followed by irradiation of a wing, viral new growths would develop about the irradiated area in similar fashion as though Rous virus circulating in the blood had localized in the slightly injured area to infect susceptible cells and thus set off a new growth. Even though metastases generally result from transportation of infected cells, that would not necessitate the conclusion that the cells had acquired cancerous autonomy.

Now that it is possible to grow clones from single cells in tissue culture, it might be possible to demonstrate in Rous neoplasia malignant cells free from virus, indicating a situation similar to that of carcinoma originating from Shope's papilloma. Or under

suitable conditions of culture it might be possible to free infected cells from demonstrable virus and thus bring evidence that a strain of non-cancerous cells could result.

In the meantime the pathologist should maintain a wholesome scepticism respecting transmissible virus as an ultimate, activating cause of cancer (Andrews, 1950). The proliferative lesions induced by virus can be safely regarded, until proved otherwise, as viral hyperplasias, or if you prefer, neoplasias, rather than viral tumors, strictly speaking.

Discussion

Dr. Sabin: I should like to ask Dr. Goodpasture whether or not he would be willing to express an opinion as to what should guide the experimental pathologist in deciding, one way or another, whether he has produced a malignant tumor. Either he has produced it with cells altered in tissue culture, or by some method in an experimental animal to differentiate it from the various neoplasias that you have termed as being "non-malignant," or "not true cancers."

Dr. Goodpasture: I think it is eventually going to be determined on the basis of the physical appearance of the cell or the chemical reaction of cells. I do think that in experimental work one will have to set up a new set of conditions. The diagnosis of cancer is one that has come about through general agreement among pathologists from their observing the natural history of certain tumors. It isn't yet a specific cytological manifestation. It is the whole history of circumstances surrounding a particular growth. I am somewhat disturbed about this problem of the identification of cancer in relation to inoculation of experimental animals with cells in attempts to prove whether or not they might be malignant. I must confess that I can't answer your question. I think for the present it is going to depend upon cumulative evidence, like that which the pathologists have already accumulated from naturally occurring growths and which they think is reliable in the diagnosis of cancer. The experimentalists must accumulate various kinds of evidence indicating that experimental neoplasias are subject to such a classification.

Dr. Sabin: Would you, Dr. Goodpasture, accept that a metastasizing tumor produced in an experimental animal can for the

present, at least, supply the data that are required for saying that one is dealing with malignant cells?

Dr. Goodpasture: Yes, I would accept that, providing that there is no associated provocative agent that persists with the tumor.

Dr. Sabin: You would accept only such a tumor as malignant?

Dr. Goodpasture: Yes. On the same basis that one accepts the human tumor as being malignant. However, one must note that whenever you use another animal rather than the one in which the tumor originates you are changing the circumstances.

Dr. Sabin: If we are going to do experimental work trying to produce malignant tumors, to find out what they are, we've got to know when we have one.

Dr. Dmochowski: Very often we argue whether the virus is, or is not, the cause of malignancy. We know very well that when we take any carcinogenic hydrocarbon and induce malignancy that this is a malignant tumor, and there is no doubt about it in any pathologist's mind. When we look for that carcinogenic hydrocarbon, where it may be, it is gone. Yet we do not argue the point that the tumor has been produced by the carcinogenic compound. May not a similar situation arise in the case of viruses? Perhaps we look for viruses in case of malignancy at too late a stage. Perhaps we argue about transmissibility of any tumor and of malignant tumors as diagnosed by a pathologist, at a too advanced stage of development of the tumor. Perhaps we should look at what a pathologist might call a pre-malignant stage or the very early stages where a virus may be. Do you have some indication to give us? Also, would you care to express an opinion about the possibility of a virus being a causative factor of cancer —in the phase when a virus induces the malignant lesion, and in which the malignant cells proliferate without the virus being constantly present in the tumor cells? Is it necessary for the virus to be continuously present to be called the cause of cancer? Can the malignant cells, once initiated by a virus, go on multiplying without the help of a virus?

Dr. Sabin: In answering, Dr. Goodpasture, will you also define a virus for us?

Dr. Goodpasture: I have no contention with the point of view that viruses can be the provocative cause of cancer. I accept that. Perhaps if we looked earlier in some of the cancers we might find a virus that was present during this early, initial phase. My only concern is with the question whether or not viruses *per se* take part in the permanent change that takes place in cells to constitute what we call cancer.

Dr. Sabin: I would like to comment on the point Dr. Dmochowski has made. The properties of a carcinogenic hydrocarbon do not include self-reproduction. That is why even though you cannot demonstrate the presence of the carcinogenic hydrocarbon in the tumor, you don't expect to. But, we have been thinking about viruses as reproducing themselves only in their original infective form, and that is, perhaps, where we have been wrong. If we can accept the models which the bacteriophage people have supplied us, the idea that a virus must continually reproduce itself in a form in which it can be shown to be again infective is no longer tenable.

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GENETIC, HORMONAL AND AGE FACTORS IN SUSCEPTIBILITY AND RESISTANCE TO TUMOR-INDUCING VIRUSES

HOWARD B. ANDERVONT*

Many factors are involved in the maintenance or alteration of the host-virus relationships which influence the occurrence of virus-induced tumors. The purpose of this paper is to discuss briefly three factors, namely, genetic, hormonal, and age, which are known to exert a pronounced influence upon the susceptibility of experimental animals to the development and transplantation of these tumors. The reader who is interested in other factors is referred to the more comprehensive publications of Duran-Reynals (1953), Oberling and Guérin (1954), Black (1949) and Annals of The New York Academy of Sciences, vol. 54 (1952).

Perhaps the best procedure for this presentation is to describe the known effects of each factor by using typical virus-induced tumors as examples and then discussing the relationships of all three factors in several of the most extensively studied tumors. The part played by these factors in other virus infections will also be mentioned briefly.

Genetic Factors

The genetic constitution of the host determines its degree of susceptibility to infection with any virus. Resistance of bacteria to bacteriophages has a genetic basis and sensitivity to infection can be altered by mutation in the bacterial host. Webster's (1937) early studies showed that, within a species, genetic factors determined susceptibility to infectious bacteria as well as to the viruses of louping ill and St. Louis encephalitis. By well controlled selection he established two lines of mice: one susceptible and the other resistant to mouse typhoid. When tested for susceptibility to the louping ill virus the bacteria-susceptible line was relatively resistant while the bacteria-resistant line was more susceptible. He observed that within the virus-resistant

* National Cancer Institute, Bethesda, Md.

line, susceptible mice were concentrated in certain litters and this led to the development of lines exhibiting pronounced differences in susceptibility to the louping ill virus. Subsequent tests revealed these lines were also susceptible or resistant to the St. Louis encephalitis virus. Further rigid selection, extending over a period of years, led to the establishment of four lines of mice: one susceptible to bacteria and viruses; one susceptible to bacteria and resistant to viruses; one resistant to bacteria and susceptible to viruses and one resistant to bacteria and viruses. Tests of F_1 progeny showed that resistance to both bacteria and viruses was inherited in a dominant manner. Finally, he showed that lines resistant to the viruses of louping ill and St. Louis encephalitis were highly susceptible to rabies virus and concluded that susceptibility to all these infectious agents was "controlled basically by inherited factors."

Webster's excellent work has been extended by others, especially by Sabin (1952) who discovered that members of an inbred strain of mice were uniformly resistant to a strain of yellow fever virus. When these animals were bred to highly susceptible mice the resultant hybrids were resistant to the virus and Sabin showed that the genetic constitution of the resistant animals determined "the level of viral multiplication as well as the vulnerability of the tissues."

The genetic makeup of the host also determines its degree of susceptibility to tumor viruses. Black (1952) found that different species of plants show a wide range of susceptibility to the wound-tumor virus. Of special interest is the occurrence of tumors in a highly susceptible stock of sweet clover in the absence of the virus, because it resembles a situation found in an animal tumor. The occurrence of pulmonary tumors in mice is largely dependent upon hereditary factors and susceptibility to induced tumors parallels susceptibility to spontaneous growths. Strains showing the highest incidence of spontaneous tumors are most susceptible to tumors induced by a variety of carcinogenic agents (Andervont, 1955).

Lucké (1952) concluded that the virus of kidney carcinoma in frogs was species specific but Rose (1952) reported that newts are susceptible to transplanted cells of this tumor.

Considerable evidence has accumulated concerning the importance of hereditary factors in controlling susceptibility to

virus-induced tumors of fowls. Waters (1945) has developed inbred lines of chickens showing striking variations in susceptibility to the viruses of avian lymphomatosis and Rous sarcoma. Greenwood, Blyth, and Carr (1948) have also developed a line of chickens that is resistant to the Rous sarcoma virus. Their procedure resembled that of Webster's in that within a flock of chickens they had developed inbred lines for physiological characters associated with egg production. When tested for susceptibility to the Rous virus one of these lines proved to be relatively resistant and the progeny of one bird were particularly resistant. Further inbreeding of these progeny established the resistant line in which "almost all gave either very small tumors, or more often, complete regressions." These resistant chickens attain added significance since filtrates of the Rous sarcoma have induced tumors in pheasants (Andrewes, 1932, 1933), ducks (Duran-Reynals, 1942) and guinea fowls (Duran-Reynals, 1943), while tumor cells or unfiltered extracts have produced tumors in pheasants (Andrewes, 1932), ducks (Duran-Reynals, 1942), turkeys and guinea fowls (Duran-Reynals, 1943), as well as the anterior chamber of the guinea pig eye (Shrigley, Greene, and Duran-Reynals, 1945). Time and the primary purpose of this paper do not permit discussion of the interesting observations accompanying heteroinoculation of the Rous sarcoma virus.

Genetic factors in susceptibility to virus-induced tumors of mammals have received most attention. It is well known that the Shope papilloma virus has, as its natural host, the Western cottontail rabbit, but it can be transmitted to certain other wild rabbits and to domestic rabbits (Syverton, 1952). The Shope fibroma virus is transmissible to domestic rabbits (Smith, 1952). Kilham (1955) has recently found that wild squirrels develop cutaneous nodules which bear histological resemblance to the Shope fibroma and neutralization tests suggest a relationship between the squirrel and rabbit viruses. Extracts of squirrel lesions have produced the disease in squirrels and in domestic rabbits. Squirrel fibroma was carried through four passages in woodchucks and then for two passages in domestic rabbits. Mosquito transmission from squirrel to squirrel was accomplished under laboratory conditions.

The virus of rabbit myxomatosis is similar to that of the Shope fibroma in that it has been transmitted only to rabbits and, from

her studies of the transformation of fibroma virus into myxoma virus, Smith (1952) stated that "host factors are at work of which we have as yet no clear understanding."

The importance of genetic factors in virus-induced tumors are best understood in the laboratory mouse, for the simple reason that highly inbred strains of these animals are available which permit the investigator to evaluate the influence of a virus upon animals of known genetic constitutions (Andervont, 1955). An inbred strain is obtained by brother-to-sister matings until all individuals are genetically identical. In mice this requires at least 20 generations of inbreeding after which we have strains exhibiting high or low susceptibility to the development of tumors and a strain uniformity in response to carcinogenic stimuli. The use of inbred strains reduces individual variations to the minimum.

Such strains of high and low susceptibility to the development of mammary cancer were established by geneticists who then hybridized them reciprocally, and discovered that the hybrids showed striking differences. Those born to high-tumor strain mothers developed mammary tumors, whereas those born to low-tumor strain mothers failed to do so. Further studies in which mice were foster nursed showed that mother's milk contained a virus essential for the occurrence of mammary cancer in certain inbred mice (Dmochowski, 1953). This is a well known story but it is of considerable importance to this Symposium for studies on the hereditary aspects of this tumor led to the discovery of the virus. It should also be of more than passing interest to those in attendance that it required many years of research of one of the most extensively studied tumors in experimental animals to prove that a virus was involved in its occurrence.

Availability of hosts of known genetic constitutions not only led to the discovery of this virus but has enabled us to establish its rôle in the causation of breast cancer in mice. For example, chemical carcinogens are able to induce these tumors in inbred strains of mice which are either resistant to the virus or are susceptible to but do not carry it (Andervont and Dunn, 1950). This shows clearly that the presence of the virus is not essential for the occurrence of all breast cancers in mice. Thus, a viral etiology for one type of tumor does not prove a similar etiology for all other tumors of the same type.

Genetic factors are of much importance to the virus of mammary cancer in mice for it is known that the virus survives only in those strains of mice possessing a proper genetic makeup (Andervont, 1945a: Heston, Deringer, and Dunn, 1956). This indicates that other cancer viruses may be subject to genetic control in inbred animals, or, at least, genetic factors must receive consideration in virus-induced tumors of inbred strains.

The relationship between known genetic influences and a virus in mouse leukemia should be mentioned at this time since the viral etiology of this disease is receiving much attention and is included in this program. When mice from high and low leukemic strains are bred reciprocally their progeny show similar incidences of leukemia (Law, 1957). This result differs from that obtained with high and low mammary tumor strains and shows that mother's milk is not the vehicle for transmission of a leukemia virus. Since hybrids born to low-leukemic strain mothers and high-leukemic strain fathers do develop leukemia it is reasonable to assume that they obtain a virus from their father.

At least two routes of virus transmission from father to offspring are possible. First, the male could infect the female and she, in turn, transmit the virus to her offspring. This possibility could be explored by permitting the female to bear a second litter by mating to a low-leukemic male. If she became infected through contact with her first mate then the offspring of her second mating should also develop leukemia. Male to female transmission of the mammary tumor virus is known to occur and this easy method for detecting virus in the mother has been used successfully.

Second, the young could be infected directly by their father's virus while in utero and not necessarily transmit the virus to their mother. Confronted with this problem there are two obvious approaches. One is to test for the presence of the virus in the males by injecting the contents of their seminal vesicles or ejacula into test animals. Both techniques have revealed the presence of the mammary tumor virus (Andervont and Dunn, 1948; Andervont, 1957). Another easy approach is to remove embryos from their mother's uterus and, by administration of minced embryo or embryo extracts to test mice, ascertain whether they

contain a virus. Suitable controls for all three procedures are available in males or embryos from low-leukemic strains.

Detection of a leukemia virus by these procedures would offer an explanation for the difference between incidences of leukemia and mammary tumors in hybrids born to low-tumor strain females and high-tumor strain males, but would not indicate that a leukemia virus is carried in the spermatozoa. To prove transmission of a tumor virus by way of ova or spermatozoa requires the production of tumors by administration of ova or spermatazoa to test animals. Detection of a virus in embryos, ovaries, or testes of infected animals is insufficient evidence that it is "egg-borne" (Gross, 1955; Gross, 1956).

It is hoped that this brief discussion will show how inbred animals can be used to study possible routes of transmission of tumor viruses and to establish the viral etiology of a tumor. Results obtained with a virus must be compatible with known genetic factors. Recognition of these factors gives the investigator an enormous advantage by knowing in which strains he may or may not expect to find a virus and which strains may be most suitable as test animals for the presence of a virus.

Age Factors

It has been known for years that age modifies the host's response to infection with many viruses. Young plants are often more susceptible than older plants, while with animal viruses the young are either more susceptible than adults or develop different symptoms. Age is of special importance when a virus is introduced into species other than its natural host. Thus, newly hatched chicks are more susceptible than full grown chickens to cutaneous, corneal or intracranial inoculation with vaccine virus (Andervont, 1927), and 14-day-old mice are more susceptible than adults to administration of herpetic virus (Andervont, 1929a). Perhaps the most striking example is the susceptibility of suckling mice to Coxsackie viruses when adults are almost completely resistant.

The age of the host is often crucial in heterologous transmission of viruses and most successful efforts to overcome species resistance to tumor viruses are dependent upon the use of young experimental animals. Andrewes (1932) found that "half grown

or adult" pheasants could be infected with the Rous sarcoma virus. He (1933) also described the production of tumors in two 7½-week-old pheasants with the same virus: one tumor was carried through three generations of pheasants by the use of tissue transplants. Duran-Reynals (1942, 1943) has conducted the most extensive efforts to transmit Rous sarcoma virus to other species and found that only 1-day-old ducks were susceptible to filtered tumor extracts, while in guinea fowls, filtrates evoked tumors in one to 14-day-old birds but failed to do so in those that were 38 days of age. Unfiltered extracts produced tumors in all of five turkeys that were one to 25-days-old and in six of ten that were 45 to 75 days of age.

Age factors in susceptibility of natural hosts to tumor viruses is of interest and could be of considerable significance. Burmester and Denington (1947) used 1-day-old chicks in their successful efforts to transmit naturally occurring chicken visceral lymphobatosis with cell free materials. Burmester (1952) inoculated five age groups of chickens with a filtrate from a transplanted strain of visceral lymphomatosis and in doses proportional to the size of the animals. The incidence of resultant tumors decreased from 95 per cent in 2-day-old chicks to 31 per cent in 114-day-old animals. When virus free chicks were exposed to infected birds, chicks kept in isolation for 30 days before exposure showed a higher incidence of lymphomatosis than those isolated for 60 days. Chicks are not only more susceptible than full grown chickens to the virus of Rous sarcoma but they also respond with different lesions (Duran-Reynals, 1940).

Duran-Reynals (1945) showed that young domestic rabbits were more susceptible than adults to extracts of Shope fibroma and in newborn rabbits administration of extracts produced widespread lesions resembling those found in adults infected with myxoma virus. Kilham (1955) transmitted squirrel fibromas to suckling squirrels whereas adults developed temporary lesions. The virus of rabbit myxomatosis is so lethal to both young and adult hosts that accurate determinations of age factors in susceptibility are very difficult.

Conflicting results have attended studies on age susceptibility to the rabbit papilloma virus. Rous and Beard (1934) found very young domestic rabbits more resistant than adults but their findings were not confirmed by Fischer and Syverton (1951).

Mice display a striking age difference in susceptibility to the mammary tumor virus in that suckling mice are far more susceptible than adults (Andervont, 1945c). Of more interest is the response of mice to inoculation with leukemia extracts. Gross (1955) has emphasized the use of newborn mice less than 12 hours old for detection of a virus which, in his experience is almost incapable of infecting adult mice.

Table 1 is an attempt to summarize age factors in the susceptibilities of the nine tumor-inducing animal viruses discussed here.

TABLE 1

Properties of Nine Tumor-Inducing Viruses

Natural Host	Tumor	Host Susceptibility		Incubation Period	Tumor Site Following Inoculation		Vector
		Young	Adult		Local	Remote	
Frog	Renal carcinoma	?	+	Months	—	+	?
Chicken	Visceral lymphomatosis	++	+	Months	+	+	Excreta Egg
Chicken	Rous sarcoma	++	+	Days	+	—	—
Rabbit	Papilloma	+	+	Days	+	—	Contact? Insect
Rabbit	Fibroma	++	+	Days	+	—	Contact? Insect
Squirrel	Fibroma	+	±	Days	+	+	Insect
Rabbit	Myxoma	+	+	Days	+	+	Contact? Insect
Mouse	Mammary carcinoma	++	±	Months	—	+	Milk
Mouse	Leukemia	+	—	Months	—	+	?

Plus marks in the second column denote qualitative differences only. The third column summarizes the time required for lesions to appear after introduction of virus or tissue extracts. Precise data are not given and the terms days and months are used to express wide variations in incubation periods. The fourth column shows differences between the viruses in their tendencies to elicit tumors at or remote from the site of administration. The last column includes routes of transmission under natural or laboratory conditions. Apparently the virus of Rous sarcoma is not contagious while spread of viruses from rabbit papilloma, fibroma and myxomatosis through contact alone is suspected but not proven. Insect transmission of rabbit papilloma (Dalmat,

1957) and fibroma (Kilham and Dalmat, 1955) as well as of squirrel fibroma (Kilham, 1955) has been accomplished under laboratory conditions. Epidemiological and laboratory studies of the spread of rabbit myxomatosis have established insect vectors (Fenner, Day, and Woodroofe, 1952; Andrewes, Muirhead-Thompson, and Stevenson, 1956).

Table 1 contains information other than age factors in susceptibility to tumor-inducing viruses. It is presented to show the wide range of biological properties displayed by these viruses in the belief that the diversity of this group of viruses should be emphasized early in this Symposium.

Age factors in susceptibility to viruses probably reflect the inability of newborn and young animals to produce antibodies, or, with the viruses of chicken lymphomatosis and mouse mammary tumor, the young are highly susceptible to a virus which remains quiescent for months before tumors occur. Resistance of very young animals to virus infections is often associated with passive immunity acquired from the mother (Andervont, 1929b).

Hormonal Factors

Recent studies with cortisone have revealed the pronounced effect of this hormone upon virus infections (Sabin, 1952; Horsfall, 1952). Probably the most striking result of cortisone administration is the alteration of susceptibility to Coxsackie viruses; normally resistant adult mice become as susceptible as suckling mice. Cortisone also has a strong enhancing influence on poliomyelitis virus in hamsters and monkeys where it supports an increased level of viral multiplication.

Hormonal stimulation is important in many forms of cancer including some virus-induced tumors. Braun (1952) suggested that hormonal influences are important in the development of the cells of crown-gall tumors. Lucké (1952) found more frog kidney carcinomas in males. Burmester (1945) observed a higher incidence of chicken visceral lymphomatosis in females. A limited survey of the literature failed to locate any impressive reference to sex difference in the response of chickens to the Rous sarcoma virus or to the occurrence of squirrel or rabbit virus-induced tumors. A reference was found to the enhancing effect of cortisone on the lesions of rabbit fibroma (Smith, 1952).

Extensive studies have been made of hormonal factors influencing leukemia and mammary tumors in the mouse, and there are several good reasons for such studies. (1) The tumors arise spontaneously in a common laboratory animal. (2) They usually occur spontaneously in middle or late life or, when induced with viruses, the incubation periods extend over months. (3) They do not appear at the site of inoculation; a characteristic which could make them more responsive to hormonal activity than those tumors occurring promptly at the site of administration. (4) Of most importance, they are studied in inbred strains of animals showing a high or low incidence of tumors and in which the relationships between virus, genetic factors, and hormonal factors can be accurately determined.

Inbred strains showing a tendency toward the development of spontaneous leukemia are available and in some of these strains the disease occurs more frequently in females (Law, 1957). Furthermore, in many strains administration of estrogens increases the incidence of leukemia and large doses of estrogens are most effective. Of particular interest to this Symposium is the fact that estrogens can provoke leukemia in strains showing a low incidence of spontaneous tumors. Simultaneous administration of androgens and estrogens inhibits the leukemic action of the latter. Testosterone propionate inhibits the spontaneous disease in one strain and castration of males frequently leads to an increased incidence. Adrenalectomy results in an increased incidence of leukemia in some strains.

Various types of radiation and chemical carcinogens induce leukemia in inbred strains of mice and it is known that in some strains estrogenic stimulation augments the action of one or both of these agents. Time does not permit discussion of the importance of the thymus gland in the occurrence of spontaneous and induced leukemia of inbred mice beyond the established fact that thymic tissue is essential for the development of the disease in most strains.

For the purpose of this discussion the role of hormonal stimulation and other factors in the genesis of mouse leukemia is just as important as genetic factors. Thus *all* these factors must be correlated with the activity of a virus before the viral etiology of the disease can be understood. This is not an impossible under-

taking for years of research have established the rôle of the virus in the causation of breast cancer in mice.

Before the discovery of the mammary tumor virus it was known that estrogenic stimulation was essential for the occurrence of breast cancer in mice. Within an inbred strain showing a tendency to develop this type of tumor, castration of females at an early age prevents the appearance of tumors, while their male litter mates develop tumors when exposed to estrogenic stimulation. Indeed, the interplay of virus, genetic factors and hormonal factors in the production of these tumors has been a fascinating problem (Andervont, 1955). Many interesting observations have been made during the course of these studies but the one most pertinent to this discussion is the ability of estrogens to producce tumors in the absence of the virus (Andervont, 1945b).

Following the observation that, given a proper genetic constitution, adequate hormonal stimulation can elicit mammary tumors in the absence of the agent, we tested various strains and F_1 hybrids to ascertain their susceptibilities to these induced tumors. Virus-free male mice two months of age were castrated and given, subcutaneously, 5 mg. stilbestrol-cholesterol pellets containing 20 per cent stilbestrol. Mice from one inbred strain and F_1 hybrids derived from this strain and two other strains exhibited tumor incidences ranging between 54 and 84 per cent. In this study the susceptible strain and parents of susceptible hybrids were all susceptible to the virus. However, other virus susceptible strains were resistant to estrogen-induced tumors. Thus it was shown that, in addition to chemically induced tumors mentioned previously, hormonal stimulation can also produce mammary tumors in virus-free mice.

For our purpose these results again emphasize that the viral etiology for one type of tumor should not lead to the assumption that a virus is responsible for all other tumors of the same type.

Discussion

Genetic, hormonal and age factors are important in the occurrence and growth of many tumors in experimental animals. The fact that the influence of these factors is not limited to virus-induced tumors indicates that tumors of known viral etiology

should not be considered as belonging to a distinct or peculiar group of neoplasms. Indeed, the diversity among virus-induced tumors is sufficient to include all types of experimental tumors.

The genetic constitution of the host is extremely important in cancer research and influences capable of surmounting genetic factors in the transplantation of tumors are also effective with virus-induced growths. Age is one such influence, for transplantable tumors will often grow in young hosts when adults are almost completely resistant. Young animals are of particular importance in the propagation of tumors in homologous or heterologous recipients. This receptivity of very young animals to the growth of foreign tumor cells and viruses is also found, perhaps to a greater degree, in the embryonated egg. Furthermore, it is known that tumor cells grow better and survive longer when implanted into the brain or anterior chamber of the eye of foreign hosts. All three techniques, age, egg, and inoculation site, can be used to compare virus-induced tumors with other neoplasms and to search for viruses in tumors of unknown etiology.

A feature of certain tumor viruses is their ability to provoke different lesions in young and adult heterologous hosts. In addition, administration of the Rous sarcoma virus to very young foreign hosts has resulted in modification of the virus so that it not only elicited different lesions but it also changed its affinities for both tissues and species. It would be of interest to know whether other tumor viruses react in a similar manner when exposed to young heterologous hosts. Since young animals are usually more susceptible than adults to infection with viruses, such studies may reveal whether tumor viruses possess any unique or common characteristic which makes them distinct, or whether their ability to undergo modification is similar to many other viruses.

It may also be of interest to know whether cells from tumors of unknown etiology become altered through exposure to the tissues of heterologous hosts. We are speculating now of the possibility that tumor cells differ from normal cells in that the malignant cells resemble viruses, and that this quality could contribute to their ability to undergo continuous proliferation in new hosts.

Hormonal factors are essential for the development of certain virus-induced tumors and others of unknown etiology. They are also essential for successful propagation of certain non-viral tu-

mors in isologous hosts, but I am not aware of a virus-induced growth that is dependent upon hormonal stimulation for successful growth in such hosts. However, the number of known virus-induced tumors is too small to justify any further emphasis of this difference.

Cortisone increases host susceptibility to heterologous transplantation of tumor cells and to virus infections. This recent development in both cancer and virus research could be of considerable assistance in the search for viruses in tumors of unknown etiology or of suspected viral etiology.

The specificity of tumor viruses has been one of the major obstacles facing those who attempted to detect a virus in human tumors. Further studies of the response of newborn animals to infection with tumor viruses and the modification of the viruses in these animals, together with further studies of increased susceptibility of cortisone-treated animals to growth of heterologous tumor cells and viruses may produce results which will ultimately lead to the detection of tumor viruses through the use of heterologous animals. Hormonal influences other than cortisone, or hormonal influences plus other factors such as radiation, may be more effective than cortisone. In brief, the growing ability of investigators to surmount genetic factors by the use of newborn animals and by hormonal stimulation may enable them to transmit tumor viruses of one species to another species.

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IMMUNOLOGIC FACTORS IN VIRAL INFECTIONS*

With Comments on Their Bearing on Certain Cancer Problems

EDWIN H. LENNETTE

The term "immunity" refers to the ability of an organism to resist invasion of the body or body cells by parasites and thus, in those cases where the parasite is a pathogen, to resist the development of disease. Under the broad designation "immunity" is included not only acquired immunity but also what is referred to as natural immunity or, sometimes, natural resistance. Acquired immunity has its basis in a prior exposure of the susceptible host to a living or dead pathogenic agent or its poisons. Natural immunity, on the other hand, has its basis not in immunologic mechanisms established by prior infection, but in such factors as species, sex, nutritional state, hormonal balance, age and genetic constitution. Both forms of immunity play a role in the total picture of resistance to infection even though the term "immunity" is apt to carry, to the microbiologist, connotations primarily of acquired immunity and, to the oncologist, connotations primarily of natural immunity.

Although the foremost concern of this conference is with the role of viruses in oncological problems, I should like for comparative purposes to consider some of the effects of genetic, hormonal, and age factors on susceptibility or resistance to viral agents other than those which produce tumors.

Evidence that susceptibility or resistance to infection by a virus is a heritable factor was first adduced only twenty years ago by Lynch and Hughes (1936) and by Webster (1937). Webster's experiments showed that the susceptibility or resistance of "virus-susceptible" or "virus-resistant" strains of mice was not universal to all viruses, but only to a specific virus or group of viruses. Some clarification of the mechanisms on which inherited resistance to viral infections is based has recently been effected by Sabin (1952). Sabin noted that unlike Swiss mice, which are

* From the Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley.

completely susceptible to the 17-D yellow fever virus, the PRI strain of mice (albino) is uniformly resistant to this virus. The resistant state is a reflection of the inability of the virus to multiply in the brains of the PRI mice to the very high levels attained in the brains of the highly susceptible Swiss mice. Resistance was found to be dominant, and referable to a single pair of genes which inhibit multiplication.

However, this heritable resistant state may, under certain conditions, be overcome. Thus, newborn PRI mice are uniformly susceptible to the 17-D virus despite the fact that the level to which the virus multiplies is no greater in the fatally-infected young animal than it is in the mature, resistant animal. Also, while adult PRI mice are completely resistant to the 17-D virus, a variable proportion succumb to inoculation with the French neurotropic strain of yellow fever virus. The fatal infections bear no obvious relationship to the amount of virus injected and there is no difference in the concentration of virus present in the brains of mice which succumb and of those which survive. *In toto*, these findings indicate that resistance based on suppression of viral multiplication may be negated by the existence of a heightened cellular susceptibility. Sabin (1952) has shown that cellular vulnerability is genetically determined, and that the factors which suppress viral multiplication and those which enhance cellular vulnerability are inherited independently.

The genetic factor in PRI mice which suppresses viral multiplication affects not only the 17-D yellow fever virus but also certain other viruses. However, some viruses of this latter group, unable to gain a foothold because their reproduction is restricted by this genetic factor, may circumvent this difficulty through the development of mutants or variants which possess a high pathogenicity in relation to their low concentration, or are able to multiply to a very high level because they have acquired the ability to by-pass the mechanism that normally depresses viral growth.

There is every reason to believe that mutants and variants appear continuously in viral populations, just as they do in bacterial populations. The new tissue culture methods now so extensively employed in virologic studies should help to shed much light on this aspect, but even without these newer tools it has been possible to produce, through proper manipulation of the environment in which growth occurs, mutants possessing the prop-

erties which the investigator desired to bestow on the virus. With respect to the situation under discussion here, mutants may be produced which are able to grow freely in host cells that ordinarily will permit the parent virus to grow only poorly, or not at all. Instances of this are the neurotropic strain of influenza virus, a mutant which will grow in the mouse brain; the dengue viruses, adapted from growth in the embryonated egg to growth in the mouse brain and the hamster brain; and the poliomyelitis viruses, adapted from growth in the mammalian CNS to growth in the murine CNS. The appearance of variants with the capacity to grow in the presence of chemicals which inhibit or deter the growth of the parent strain has been described; Ginsberg and Horsfall (1949a), for example, obtained from mumps virus a variant which grew freely in the presence of large amounts of capsular polysaccharide from a bacterium (Friedlander's bacillus) although the parent strain would not grow in such an environment.

Such variations undoubtedly afford a very important means for survival of the virus in nature as, for example, in the face of a highly immunized host population. The presence of specific antibody in a large part of the host population constitutes an obstacle to the dissemination (and thus, perpetuation) of a particular virus; it does not necessarily mean that the agent will become extinct because of a lack of susceptibles. The immunologic environment in which the virus finds itself may stimulate the emergence of variants or mutants able to survive in the unfavorable milieu. Persistent antigenic variation of influenza virus has been reported (Archetti and Horsfall, 1950) during the course of chick embryo passage of virus partially neutralized by heterologous antiserum; similar experiments utilizing homologous antiserum rather than heterologous antiserum have also given rise to variants (Isaacs and Andrewes, 1951). Magill (1955) showed that the influenza virus can survive, and can be propagated in immunologically adverse environments, *i.e.*, in mice vaccinated with the strictly homologous strain of virus. Survival of the virus was associated with the emergence of variants which differed antigenically from the parent strain. Difficulty was encountered in establishing the passage series whenever the immunologic environment was changed. However, once the passage series was established, *i.e.*, the virus was adapted, its

multiplication apparently occurred to the same level in the vaccinated mice as in the unvaccinated control animals. This is of interest in connection with what was said earlier concerning the genetically-transmitted factor which suppresses viral multiplication in PRI mice, and its contravention by variant strains with the capacity to multiply to very high levels. Changes in the antigenic constitution of the influenza viruses occur in nature. The changes, through the continuous evolvement of variants, are progressive although, at any one interval of a long temporal span, relatively minor in degree. The cumulative effect of these gradual changes, however, is such as to make two strains, separated from each other by an appreciable span of time, appear notably different in their antigenic composition. This evolutionary process is sometimes referred to as "immunological drift."

Resistance to infection is also based on factors related to age. The resistance acquired with age is often spoken of as "maturation resistance" or "maturation immunity," and consists of at least two types.

In the first type, demonstrable in the mouse, there develops with age an increased resistance to peripheral inoculation with neurotropic viruses (Lennette and Koprowski, 1944; see references, Lennette, 1954a). Such viruses are obliged after peripheral inoculation, to follow neural pathways which eventually give them access to the central nervous system and the susceptible cells for which they have a predilection. The development of resistance apparently reflects the development of barriers which impede movement of the virus along these pathways. These barriers may be highly localized (myoneural junction, retinal neurons, anterior rhinencephalon, etc. (Sabin, 1952)), and appear at different ages, depending upon the host, the pathogenetic properties of the particular virus, and the pathway utilized (see summary by Lennette, 1954a).

In the other type of maturation resistance, the central nervous system itself is refractory to infection. In this type, which has been studied primarily in the rat, the CNS undergoes, with increasing age, some subtle change whose end result is to keep viral multiplication depressed to a low level, a level which does not permit the virus to exert its lethal potentialities. Studies with the St. Louis virus, for example, have shown (Sabin, 1952) that in mature rats, which are fully resistant to cerebral inoculation,

the maximal viral level attained is only 1/1,000 of that observed in the very young, fully susceptible animals, and that in animals of an age at which cerebral resistance first appears, the levels are intermediate between those observed in fully resistant and in fully susceptible animals.

Numerous experimental observations indicate that the synthesis of new virus is mediated by the enzyme systems of the infected host cells. It thus seems not impossible that the availability or non-availability of certain enzyme systems may determine, in part, the existence of a resistant or of a susceptible state. Thus, the brain of the immature rat is relatively deficient in at least three enzymes (Potter *et al.*, 1945)—succinic dehydrogenase, cytochrome oxidase and adenosine tryphosphotase. This raises the interesting question as to whether metabolic activities conducted in the comparative absence of certain enzymes are such as to afford optimal conditions for certain viruses to multiply (susceptibility), whereas the appearance with age of a full complement of enzyme systems so alters cellular metabolism that viral multiplication is suppressed (resistance). Another example of a possible relationship between susceptibility and lack of an enzyme system has recently been reported by Rowe (1953), who worked with the Coxsackie viruses. These viruses will grow in the muscles of very young suckling mice but not in the muscles of adult mice. Rowe found, however, that if the skeletal muscle of an adult animal is denervated, the virus will propagate to a considerable degree in the affected musculature. Here, too, the question arises as to whether the appearance of the resistant state is any way related to the development, with age, of two enzymes, namely, phosphorylase and phosphoglucomutase, both of which are present only in low levels in the susceptible embryonic and infant muscle. Whether there is any actual relationship between the presence of a resistant state and the presence of certain enzyme systems cannot be answered until considerably more is known of the mechanisms involved in intracellular protein and viral synthesis.

It is now generally recognized that hormonal balance is involved in resistance or lack of resistance to viral infections. In 1936, Aycock reported that the susceptibility of female monkeys to poliomyelitis was enhanced by castration and that treatment of the castrated animals with estrogens prevented the appearance

of a heightened susceptibility. Similarly, the susceptibility of the male hamster to cerebral inoculation with poliomyelitis virus is heightened by castration but this enhancing effect can be cancelled in part by administration of testosterone or gonadotrophic hormone (see Teodoru, 1956). The male hamster is completely resistant to infection with poliomyelitis virus by the intraperitoneal route, but castration renders a high proportion of the animals susceptible (Teodoru, 1956). On the other hand, it has been reported (Sprunt and McDearman, 1940) that resistance to infection with vaccinia virus is enhanced in animals treated with estrogenic hormones. Weiss *et al.* (1950) found that thyroxine decreased the resistance of mice to the murine pneumonitis virus whereas radioactive iodine, which destroys thyroid tissue and so decreases the metabolic rate, increased resistance to this virus.

The most extensive, and the most clear-cut, observations on the role of hormones in natural resistance are those dealing with the adrenocorticotropic hormone (ACTH) and the adrenal steroid, cortisone. ACTH greatly enhances the natural susceptibility of the monkey to poliomyelitis and of the mouse to the influenca virus (Ainslie *et al.*, 1951; Foster *et al.*, 1951; and Schwartzman and Fisher, 1952). On the other hand, the partial resistance of the hamster to infection with poliomyelitis virus is not appreciably affected by ACTH (Schwartzman and Fisher, 1952) but is broken down by cortisone (Schwartzman and Fisher, 1952; Sabin, 1952). Cortisone heightens the natural susceptibility of monkeys to infection with poliomyelitis virus (Sabin, 1952), and also increases the natural susceptibility of mice to infection with several neurotropic viruses (Southam and Babcock, 1951). The adult mouse, completely resistant to infection with Coxsackie viruses, can be made nearly as susceptible as the suckling mouse by the administration of cortisone (Kilbourne and Horsfall, 1951a; Sulkin *et al.*, 1952). Cortisone also enhances the susceptibility of the chick embryo to infection with the viruses of influenza and mumps; embryonated eggs treated with cortisone yield from 2 to 6 times as much virus as do untreated eggs (Kilbourne and Horsfall, 1951b). How the steroid hormones induce a normally refractory cell to produce virus or how, in a normally susceptible host, they effect so notable an increase in virus production, is unknown; the studies of Kalter *et al.* (1951) on the relation of testosterone and ACTH to influenza virus infections

suggest that these substances influence the synthesis of new virus by their effect on protein metabolism. Regardless of what the mechanism might be, the remarkable breakdown of resistance to viral infections by the steroids suggests that these powerful hormones might find application in experimental oncology. As a matter of fact, the usefulness of cortisone in this field was inquired into shortly after the hormone became available. It was shown (Billingham *et al.*, 1951), for example, that the life span of skin homografts could be lengthened by the administration of cortisone; the increased longevity of the grafts was apparently brought about through retardation of an immune response to the tissue. It has also been reported (Green and Whiteley, 1952) that administration of cortisone to rats with strongly regressing transplanted sarcomata resulted in a resumption of growth with subsequent death of the animal; the same report describes the successful transplantation of a rat sarcoma to mice, the Rous sarcoma to the pigeon, and an "oat-celled" bronchogenic carcinoma from man to mice.

Before touching on certain humoral aspects of acquired immunity, I should like to outline broadly some of our present knowledge of the mechanism of cellular infection and viral replication. Among the animal viruses, the influenza viruses have provided an excellent model for studies on viral penetration of the cell and on viral growth and I shall use these viruses as examples here because of the analogies that can be made with the bacteriophages, or bacterial viruses; the possible bearing of information derived from studies of the latter agents on the problem of the etiology of human cancer will undoubtedly be taken up to some extent by others at this Symposium.

With the influenza viruses, the initial step in infection is attachment of the virus to the host cell. The surface of the susceptible cell contains "receptors" which are mucoprotein in nature; more specifically, the receptors are comprised of conjugated proteins containing prosthetic groups made up of heterogeneous polysaccharides. The tail or terminal portion of the prosthetic groups contains a pyrrole-carboxylic acid which is the key point for virus attachment (Gottschalk, 1954). On the surface of the influenza virus particle, in turn, are certain chemical groupings or "effectors" with a pattern complementing that of

the receptors on the susceptible cell. Virus-cell union is thus a linkage of effector with specific receptor.

The second step in the infection cycle is ingestion of the adsorbed virus by the cell (viropexis). The virus possesses an enzyme (now identified as a glycosidase (Gottschalk, 1956)) with the ability to destroy receptors; destruction of the receptors, however, is not required for penetration of the virus into the cell since inactivated virus which lacks enzymatic activity can penetrate the cell. (The receptor destroying property, however, is presumably useful to the virus since it has been selected for—see below.)

Entrance of the virus into the cell is shortly thereafter followed by the period of eclipse, a period of several hours during which nothing recognizable as virus, *i.e.*, possessing viral properties or characteristics, can be detected within the cell. The somatic portion of the virus, which carries the surface antigens, the hemagglutinative properties (effectors) and enzymatic functions, is promptly broken down and since it bears no responsibility for perpetuation of the viral species, it presumably has little or no importance in the elaboration of new virus. The genetic portion of the virus, presumably represented by the small amount of nucleic acid (0.75 per cent–1.1 per cent of ribose nucleic acid) present in the virus particle, is considered, by analogy with the bacterial viruses, to be the only constituent of the viral entity capable of initiating, directing and controlling the synthesis of new virus. No infectious particles are detectable, and since there are no tests by which the presence of genetic material can be recognized, aside from the ability of the virus to multiply (infectivity), it is not possible with present biologic methods to follow any changes or alterations it might undergo. (Infectivity is apparently a function of the nucleic acid moiety of the virus particle, even though there seems to be no simple proportionality between infectivity and nucleic acid content (Ada and Perry, 1956); perhaps only virus particles with a nucleic acid content above a certain value possess infectivity. It is not impossible, however, that infectivity is determined by a specific part of the nucleic acid.) Nevertheless, the genetic material of the virus, once it has entered the susceptible cell, determines the direction in which intracellular syntheses will proceed and thereby compels

the cell to provide the materials and the energy needed to fashion the soma of the new viral particle.

The eclipse period lasts for about three hours, and is terminated by the appearance of the first evidence of viral propagation. By means of the appropriate immunologic tests, it is possible to detect the appearance and subsequent increases in concentration of non-infectious hemagglutinin and of "soluble" complement-fixing antigen, both of which are regarded by some investigators as manifestations of the presence of so-called "incomplete," *i.e.*, noninfectious, virus. (See the excellent interpretative review by Dulbecco, 1955.) Fully infective, *i.e.*, "complete" virus becomes detectable at about the fourth hour post-infection, and from this time onward, continuous liberation of virus from the cell occurs.

Maturation of the influenza virus apparently takes place near the surface of the cell (*cf.* Morgan *et al.*, 1956b). Spherical bodies of moderate density to electron beams appear at or just beneath the cell surface. This differentiation of material at the cell surface into a visible mass which represents maturing virus particles does not exclude the possibility that viral components may be elaborated within the nucleus or deep in the cytoplasm. The spherical particles, as they move out through the cell wall, acquire "an internal body, a sharply defined, dense membrane, and an envelope of less dense material" (Morgan *et al.*, 1956b). Fully mature viral particles presumably leave the cell through a process of local lysis and then remained attached to the external surface for a period of time; perhaps this terminal association between virus and cell is dissolved by the enzymatic action of the completely formed virus entity (see above). (While studies on the structure and development of influenza virus have yielded no evidence that the nucleus or adjacent cytoplasm is involved, this does not necessarily exclude the possible participation of the nucleus in the elaboration of influenza virus (Morgan *et al.*, 1956b). The nucleus has been shown to be intimately and actively involved in the synthesis of other viruses. For example, the beautiful electron micrographs of Morgan *et al.* (1956a), show that the adenoviruses develop within the nucleus and differentiate from a dense granular and reticular material into a crystal-like form. Disintegration of the crystals and disruption of the nuclear membrane releases the virus into the cytoplasm.

There is no evidence that the virus particle undergoes further differentiation in the cytoplasm.)

Experimental mixed infections with two influenza virus strains have shown that phenotypic mixing and recombination may occur. This indicates that both the genetic and the somatic components are built up of materials drawn from a common replicating pool. There is thus present the possibility that during its assembly the viral entity may have incorporated within it substances which still carry the antigenic pattern of the host, and so can be recognized immunologically as belonging to the host. As a matter of fact, this possibility has been advanced to explain the presence of normal host-cell constituents in highly "purified" preparations of tumorogenic as well as non-tumorogenic viruses (*cf.* Hauschka, 1952, page 621; and Beard *et al.*, 1955, page 167). While such explanations may eventually be substantiated, evidence in their support has yet to be obtained; until such evidence is forthcoming, normal host-tissue components present in a "purified" viral preparation must be regarded as contaminants, rather than integral parts of the virus particle. So far as I am aware, only with the virus of erythromyeloblastic leukosis has it been shown that substances bearing the host antigen pattern also form an integral part of the infectious virus unit. Beard *et al.* (1955) point out that normal chick tissue and Forssman antigens form integral parts "not only of the virus particle as a whole, but of that portion of the particle exerting the activity of the agent to transfer the disease. This relationship, so definitely evident, effectively excluded the possibility that non-specific adsorption of the antigens on the particles was a contributing factor of the phenomenon." Studies aimed at the detection or isolation of oncogenic agents from fractions derived from tissue homogenates, or concerned with antibody response to the materials in such fractions should therefore evaluate the effect that normal host-cell constituents might have in producing the results obtained.

It is not my intent here to make a case for the possible viral etiology of human cancer. The generally accepted belief that viruses have no etiologic importance in the causation of human cancer stems from the lack of evidence that human cancer is contagious and from the inability, despite numerous attempts, to isolate viral agents from affected tissues. This has lead to the

concept that, under the influence of proper stimuli, a cancer incitant might arise within the cell and produce its damaging effects without the mediation of an infectious agent. This incitant has been likened to the prophage which exists within a lysogenic bacterium and, under certain conditions, becomes essentially the precursor of bacteriophage. It will be recalled that when a phage particle infects a bacterium, the infection may proceed in one of two directions: "either the genetic material of the phage enters the vegetative phase of the cycle and phage is produced, or it becomes attached to the bacterial chromosome and is transformed into a prophage. Prophage would then be the genetic material of the phage as modified by its binding to the bacterial chromosome" (Lwoff, 1956). Normally, the prophage and bacterium co-exist harmoniously. However, under the influence of such stimuli as ultra-violet light, organic peroxides, or nitrogen mustards, stimuli which are also carcinogenic, the prophage may become converted into a lethal cellular constituent. Attempts to equate the quiescent prophage stage of bacterial viruses with the dormant phase of a latent virus infection rest on uncertain ground at the present time; latent infections with animal viruses (*e.g.*, herpes simplex, lymphocytic chorio-meningitis, psittacosis, etc.) are known to exist, and by application of appropriate stimuli the dormant infections can be made to flare up (*cf.* Koprowski, 1952), but here the analogy ends.

On the basis of the recorded literature, objections can be made to the viral etiology of cancer. I think, however, that the time has come to re-examine our attitudes and to re-evaluate and even reject some of our opinions and impressions stemming from the not inappreciable body of negative evidence on this subject. Beard, Sharp, and Eckert (1955) provided an excellent summary of the current situation when, speaking of the virus of erythromyeloblastic leukosis, they stated "the fact that it has required almost 50 years for the characterization of an agent . . . present in as simple a medium as chicken blood plasma in concentrations such that the material visibly clouds the fluid, is ample evidence that the possible presence of virus in less obvious amounts in more complex sources has certainly not been eliminated by the efforts thus far made." *Pari passu*, their statement intimates something of the formidable problems that confront the investigator who bases his experiments on the viral approach to the

etiology of human cancer, and why every possible immunologic factor has received far more consideration than have the viruses.

Attempts to detect specific antibodies against human cancers have been surrounded with similar difficulties. An infectious carcinogen, upon entering a susceptible cell, should provide it with an antigenic substance not present in the normal cell, and hence an immunologic distinction between infected and non-infected cell should be feasible. However, as recently as 1952, one reviewer concluded that "eventual demonstration of specific neoplastic antigens appears dubious, since the critical results to date have been largely negative" (Hauschka, 1952). These negative results do not necessarily prove that specific cancer antigens and specific cancer antibodies do not exist. Part of the difficulty, as Hauschka (1952) has stated, lies in the fact that "the recognition, through serologic techniques, or authentic neoplastic antigens is handicapped by the considerable array of antigenic entities known to be associated with normal cells: species- and organ-specific tissue antigens, tissue globulins, cathepsins, lipids, blood group substances and histocompatibility factors, Wassermann and Forssman antigens." Confrontation with such a spectrum of antigenic materials points to the need, in any attack on this problem, of a well-planned immunologic approach, and one which requires more than a passing acquaintance with immunologic principles and facts.

In the search for specific antigens in human cancers, crude tissue homogenates have been injected into various animal species in the hope that antibody would be produced against an antigen present in the malignant tissue but not in normal tissue. The negative results need not occasion surprise when the situation with respect to known viral agents is called to mind. Crude suspensions of virus infected tissues are used to produce infection in experimental animals and also to actively immunize animals for the production of specific antibody-containing sera. The amount of virus present in such suspensions is comparatively low. Gard (1955) has computed that even well-adapted animal strains of poliomyelitis virus seldom contain more than 10^6 infectious units of virus per gram of tissue. On a weight basis, this is in the vicinity of 0.01 to 0.1 milligrams of virus per kilogram of tissue or one millionth to ten millionths of one per cent of the wet tissue. Obviously, with such a minuscule amount of spe-

cific viral antigen, repeated injections of virus-infected tissue suspensions will be required to stimulate the production of detectable amounts of antibody in the non-susceptible animal. The large amounts of non-specific materials present in such suspensions may tie up a correspondingly large share of the antibody forming cells and thus interfere with the production of antibody to the specific material, *i.e.*, the virus, or cancer antigen. In virtually every immunologic test, except perhaps where neutralization of an infective principle is involved, it is necessary to take into account the presence of such "non-specific" antibody. Even if the unwanted antibody to normal tissue components is removed from such an immune serum through adsorption of the serum with homologous normal tissues, actual union between specific antigen and antibody may not necessarily be demonstrable. Thus, in a specific virus-antibody flocculating system, the total mass of viral particles present may be too small for the floccules formed by union of virus and antibody to be visible. This is true, for example, of the poliomyelitis viruses, which must be highly concentrated before there is sufficient mass for visible floccules to occur upon mixture of the virus with specific antibody. Similarly, in the case of the complement fixation test, the antigen may be too weak to produce visible degrees of fixation. In most instances, the amount of complement-fixing antigen present in a crude suspension of virus-infected tissue is related to the amount of infectious material originally present. We might again refer to the polioviruses. My associate, Dr. Nathalie Schmidt, studying the development of poliomyelitis complement-fixing antigens in tissue culture systems, has found that there appears to be a critical infectivity titer (ca. $1 \times 10^{-5.5}$) associated with the presence of demonstrable amounts of complement-fixing antigen; poliomyelitic tissue culture fluids with infectivity titers below this level have uniformly failed to exhibit complement-fixing activity, whereas preparations with infectivity titers above this level have usually (but not invariably) possessed fixing ability.

On the other hand, the specific immune bodies may fail to appear in the serum of an animal because the virus mass available to the antibody forming cells is too small to call forth antibody formation. Sufficient viral mass to elicit an antibody response may be lacking not only in tissue suspensions used for immuni-

zation but, of equal or even greater importance, it may even be lacking in the actively infected animal itself. The latter situation was described by Beard, Sharp, and Eckert (1955) who stated "it is evident further that the infrequent occurrence of the usual viral immune bodies in chickens with erythromyeloblastic leukosis dispels one more objection to the idea that other tumors, particularly those in man, might be of viral etiology simply because specific immune bodies have not been demonstrated in those conditions." With respect to mass, specific antibody can be elicited when sufficiently large amounts of virus are administered.

The demonstration of what appears to be a specific antibody should be regarded with caution until it can be absolutely determined that the immunologic reaction is specific. This is especially cogent when one is working in an unknown area. Non-specific reactions are due to a number of factors, some known, some unknown (Lennette, 1956), and are by no means a rarity in the complement fixation technique. Non-specific fixation of complement is more likely to occur when comparatively crude tissue suspensions or extracts are used as antigens. Furthermore, sera and tissue suspensions or extracts contain substances or components which give rise to non-specific reactions, not only in complement fixation systems but also in hemagglutination and hemagglutination-inhibition tests and in serum-virus neutralization tests. Influenza virus, for example, possesses the capacity to agglutinate chick red cells or human group "O" red cells. Since specific influenzal antibody prevents the virus from agglutinating red cells, advantage is taken of this property for purposes of quantitative antibody assay. However, non-specific inhibitors of agglutination are known to exist in human serum (and in certain animal tissues and biological fluids), and unless the existence of such interfering substances is taken into account, the results of hemagglutination-inhibition tests can be quite misleading. Non-specific effects may be mistaken for specific inhibition or the non-specific inhibitory effects may mask the presence of true antibody. In the case of influenza antibody assays, therefore, pre-treatment of the test sera with receptor destroying enzyme (RDE) from *Vibrio cholerae* filtrates may be required to remove such interfering substances.

The complexity of the situation which gives rise to non-specific

reactivity is well illustrated in the case of brain suspensions derived from mice infected with any of several different viruses. In addition to the presence of specific hemagglutinin, such infected brains contain, just as do normal mouse brains, a non-specific hemagglutinin for erythrocytes from a variety of animal species. Moreover, a thermolabile inhibitor of agglutination may be present concomitantly with these non-specific hemagglutinins.

Non-specific reactions are not restricted to purely *in vitro* systems. Normal human and animal sera have been found to possess, in varying degree, the capacity to effect non-specific neutralization of certain viruses (Ginsberg and Horsfall, 1949b). Fortunately, the factor responsible for this non-specific inactivation of virus is heat-labile; since, in its natural state, it can neutralize appreciable amounts of virus, the use of unheated sera in neutralization tests would appear, in general, to be a hazardous procedure. Under certain conditions, however, it may be desirable or even necessary to use only sera stored in the frozen state (to protect this labile factor) or to add fresh unheated sera to test sera which have been previously inactivated. This is because effective neutralization of virus in some instances occurs only in the presence of two factors, *viz.*, a specific heat-stable antibody together with a non-specific heat-labile accessory substance, or because the addition of fresh serum has a potentiating effect on the specific neutralizing capacity of the test serum. The nature of the non-specific virus-inactivating substance present in human and mammalian sera has recently been investigated by Karzon (1956) and the evidence indicates that the inhibitor is similar to or identical with the properdin system. Thus, the activity of the viral inactivating substance is abolished by trypsin and streptokinase, presumably as a result of the destructive action of these enzymes on complement in the serum. Ethylene diamine-tetra-acetate, which has a strong chelating action on polyvalent metal ions, apparently destroys the viral inactivating substance by binding magnesium ions. Inability to restore the activity of the inactivated viral inhibitor by adding magnesium may be due to the adverse effects of the increased ionic strength resulting from the added magnesium; inhibition of the properdin system is known to occur under such conditions. An inhibitor of the viral inactivating substance is widely distributed in the tissues, and is associated with the nuclear, mitochondrial and micro-

somal fractions; its activity seems attributable to the presence of high molecular weight polysaccharides which have been shown to combine with properdin (Pillemer and co-workers, cited by Karzon, 1956). It is of interest that, as Karzon (1956) points out, "the circulating antiviral system, together with the counter systems in the blood and the tissues, presumably could play a role in so-called 'species' or 'natural' susceptibility or resistance."*

It is encouraging to note that despite the many obstacles and difficulties, such as those delineated above, attempts are still being made to uncover a specific cancer antigen. It is to be hoped that these sporadic inquiries can be expanded into a broader, and more continuous, effort and that as new immunologic methods or tools are developed, they will be applied to the cancer antigen problem. Some of the most recent efforts on this problem might be mentioned. Seligmann *et al.* (1955) undertook a comparison of the antigenic make-up of normal and leukemic cells using the recently developed gel diffusion techniques. Analysis of the complex precipitation patterns led them to conclude that leukemic cells lack an antigenic factor which is present in normal leucocytes. Weiler (1956), making a comparative antigenic analysis of malignant and of normal liver cells by means of a complement fixation technique, also concluded that the malignant cells lack an antigen present in normal cells. In a recent study, Bjorklund (1956) compared normal tissues with malignant epithelial tumors from various sources by means of gel diffusion analyses. His findings by this technique agree with the previous work of others, who used different methods; he noted that certain tumor cells possess antigenic components in common with certain normal cells. Also, he found, unlike Weiler and Seligmann *et al.*, "that several . . . normal human tissues from which tumors . . . frequently develop lacked one distinct antigenic property found in human cancer tissue." Using alcoholic tissue extract as complement-fixing antigens, Kobayashi (1956) too, reported that his extracts contained antigens specific for human gastric cancers. The greatest antigenic activity and the highest specificity resided in the nucleic acid fraction obtained

* After this paper was completed, there came to our attention the report of Wedgwood *et al.* (Wedgwood, R. J., H. S. Ginsberg, and L. Pillemer: The Properdin System and Immunity. VI. The Inactivation of Newcastle Disease Virus by the Properdin System. *J. Exp. Med.*, 104: 707–726, Nov., 1956) which identified the viral inactivating substance with properdin.

from the cancer (Kobayashi and Kawasaki, 1956). These reports illustrate, without the need for citing additional literature, that investigations directed at the same objective have led to diametrically opposite findings. The significance of such results is indeed difficult to evaluate, and one can only conclude that, at the present moment, no unequivocal and indisputable evidence has been brought forward to show that the human cancer cell possesses an antigen or antigens in addition to those present in the normally-functioning cell. Nevertheless the findings from the immunologic standpoint are provocative, and the importance of the problem in itself demands continuing investigation.

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FACTORS INFLUENCING THE PROLIFERATION OF VIRUSES*

JOSEPH L. MELNICK, G.-D. HSIUNG, CATHERINE RAPPAPORT, DAVID HOWES, AND MAGDALENA REISSIG

The many papers which appear each month attest to the extensive knowledge of certain details of virus multiplication, and yet the over-all picture is far from complete. Each laboratory working with animal virus has had to take into consideration the topic of this paper, *Factors influencing the proliferation of viruses*, and in the present discussion we shall review some of the recent experiences of our laboratory in this field. This paper will be concerned with:

(1) the adsorption and growth of virus in cells of differing susceptibility,

(2) the maturation and release of virus from cells grown in serum-containing and in synthetic media, and

(3) the effects of cell nutrients on cytopathic responses.

Cell Susceptibility and Resistance

As models for this study, kidney monolayer cultures of rhesus (*Macaca mulatta*) and red grass (*Erythrocebus patas*) monkeys were used. Patas cells had been found to be about two to three times as sensitive to poliovirus as rhesus cells, but much less sensitive to Coxsackie A9 and to the group A echo viruses (see table 1) (Hsiung and Melnick, 1955, 1957b).

Dr. Hsiung has carried out some recent experiments to determine (a) whether the greater number of poliovirus plaques appearing on patas cells was due to variations among the virus particles in respect to their rate of adsorption, or to their ability to produce plaques in the two species, or (b) whether the variation in production of plaques reflected differences in the number of susceptible cells present in cultures of the two species.

The following experiments were performed:

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TABLE 1

Susceptibility of Rhesus and Patas Cells to Enteric Viruses

Virus	Virus Conc.	Rhesus		Patas	
		Avg. No. Plaques Per Bottle	Log. Titer PFU/ml	Avg. No. Plaques Per Bottle	Log. Titer PFU/ml.
Polio-1	$10^{-5.7}$	8.9	7.6	23.5	8.1
Cox. A9	$10^{-6.7}$	18.5	8.9	0	
	$10^{-1.0}$	..	..	0*	< 1.0
Cox. B1	$10^{-4.3}$	17	6.5	33.5	6.8
Echo-1 (Group A)	10^{-6}	24.2	8.4	0	
	10^{-1}	..	..	0*	< 1.0
Echo-7 (Group B)	10^{-5}	25.3	7.4	23	7.4

* With cultures from certain lots of patas monkeys about 50 tiny, delayed, slow-growing plaques have appeared at this concentration.

Single adsorption of poliovirus on rhesus and on patas cells. Aliquots of virus containing approximately 10–20 rhesus plaque-forming units (PFU) were introduced into five bottle cultures each of rhesus and patas. After two hours' adsorption at 37° C., non-adsorbed virus was measured (by combining the supernatant fluid from the adsorption bottles with two washing fluids of 0.5 ml. phosphate-buffered saline (PBS), and inoculating them into fresh bottle cultures of either rhesus or patas cells. After an adsorption period of one hour, the second group of cultures used for assay were overlaid with nutrient agar. Results are shown in table 2.

It will be noted that the susceptibility ratio, S.R. (Number of plaques in patas cultures/Number of plaques in rhesus cultures),

TABLE 2

Comparison of Poliovirus Adsorption on Rhesus and Patas Cells at 37° for 2 Hours

	Virus Adsorbed (PFU)*	Free Virus Plated on Patas (PFU)*	Free Virus Plated on Rhesus (PFU)*	Per Cent Adsorption†
Patas	100	35	(15)	74
Rhesus	60	(60)	30	67

* Total plaques in four bottles.

† $\frac{\text{Virus adsorbed}}{\text{Virus adsorbed plus free virus plated on same species}}$

was about 2, and was not significantly different with particles in the original inoculum (100/60 or S.R. = 1.7) or with particles obtained after adsorption on patas cells (35/15 or S.R. = 2.3) or on rhesus cells (60/30 or S.R. = 2). This indicates that the relative susceptibility of the cells themselves was responsible for the difference in plaque production in patas and rhesus cultures. Regardless of whether the non-adsorbed virus particles were removed from rhesus or from patas cells, the number of plaques appearing on patas was always higher than on rhesus.

No significant difference was found in the percentage of virus particles adsorbing on patas or on rhesus cells. This experiment was repeated several times, and the results were found reproducible.

Similar experiments were carried out in which virus was subjected to three successive adsorptions on cells of both species and, after each adsorption, the susceptibility ratio was determined, using the non-adsorbed free virus as the test virus. The susceptibility ratio, as determined from data such as shown in table 2, showed no marked difference in the original particles or in those obtained after three adsorptions on cells of either species. If there were particles in the original preparation which had the property of adsorbing only on patas cells, then after three successive exposures to rhesus, the patas-adsorbing particles would be enriched. One would then anticipate exceedingly high adsorption when the particles which failed to adsorb on rhesus were plated on patas cells, and very low counts on subsequent exposure to rhesus cells, *i.e.*, a very high susceptibility ratio. But as shown in table 3, after the third adsorption the susceptibility ratio was

TABLE 3

Susceptibility Ratio of Patas and Rhesus Cells, Using as Seed Poliovirus Exposed Serially to Cells of Each Species

Original virus	Susceptibility Ratio* 1.9	
	After Adsorption on Patas	After Adsorption on Rhesus
First adsorption	1.9	2.3
Second adsorption	1.9	1.6
Third adsorption	1.9	1.5

* $\frac{\text{No. of plaques on patas monolayer}}{\text{No. of plaques on rhesus monolayer}}$

1.5. On the other hand, virus particles which had been seeded serially three times on patas cells, with total adsorption periods of 6 hours, might be expected to give higher adsorption on rhesus than on patas, or at least equal adsorption on both species. Yet the final susceptibility ratio was 1.9, the same as that found after the first two adsorption periods. Thus these results point to enhanced cell susceptibility and not to variation within the virus population as the factor in the increased plaque formation on patas monolayers.

Comparison of adsorption kinetics of poliomyelitis, Coxsackie A9, and echo-1 viruses. Earlier studies from our laboratory (Kaplan, 1955) had shown that monolayer cultures of the resistant capuchin species *(Cebus capucina)* failed to adsorb measurable amounts of poliovirus. In contrast, Dr. Hsiung has been able to show that the resistance of patas cells to Coxsackie A9 and echo-1 viruses is not due to a failure to adsorb virus particles, and that the cause must lie in some other factor of the infection cycle.

Aliquots of each of these viruses, containing 20–40 PFU, were inoculated into 20 rhesus and 20 patas bottles and incubated at 37° C. At 15, 30, 60, and 120 minute intervals, four bottles from rhesus and four from patas were taken from the incubator. The supernatant fluid, containing non-adsorbed virus particles removed either from patas or from rhesus, was seeded into fresh bottle cultures of rhesus cells, and after a further adsorption period of 2 hours, agar was added. The original bottle cultures were overlaid immediately after removal of the supernatant fluid, to determine the amount of virus adsorbed. At the end of 120 minutes, four bottles from each group were treated as above and the other four were overlaid without removing the supernatant fluid. The number of plaques in the last group of cultures represented the total virus inoculated. Table 4 gives the comparative results of the adsorption of the three viruses. Coxsackie A9 and echo-1, to which patas cells are apparently resistant, were adsorbed on patas cells as well as on rhesus although plaques appeared only on rhesus cells. The non-adsorbed free virus from patas was assayed by plating it on rhesus cells. Poliovirus, which yielded a higher plaque count on patas cells than on rhesus, was adsorbed by patas to about the same degree as were Coxsackie A9 and echo-1 viruses. Obviously the difference between the

TABLE 4

Rate of Adsorption of Poliovirus, Coxsackie, and Echo Viruses on Rhesus and Patas Cells

Virus	Adsorption Time (min.)	Average No. Plaques on Rhesus Bottles			Average No. Plaques on Patas (1st Adsorp.)	Per Cent Adsorption on Rhesus*	Per Cent Adsorption on Patas
		Virus Ads. on Rhesus (1st Adsorp.)	Free Virus From Rhesus	Free Virus From Patas			
Polio–1	30	8.3	8.3	8.7	13.0	61	59
	60	10.5	6.0	5.3	18.1	71	75
	120	20.3	4.8	2.5	30.3	77	88
	120	21.0	not removed				
Coxsackie A9 .	15	26.8	12.0	13.2	0	63	67
	30	38.3	8.6	7.3	0	79	82
	60	32.2	8.8	7.3	0	78	82
	120	40.0	not removed				
Echo–1	30	34.0	7.0	8.6	0	83	79
	60	38.8	2.2	4.6	0	94	89
	120	40.2	5.0	4.2	0	88	90
	120	40.0	not removed				

* Per cent adsorption = $\frac{\text{Total Virus (}i.e.\text{, rhesus PFU at 120 min. in cultures where virus was not removed) minus Free Virus}}{\text{Total Virus}}$

high susceptibility of patas to poliovirus and its extremely low susceptibility to the Coxsackie and the echo virus must be due to some factor other than adsorption of virus particles to the cell.

Virus mulitplication and cytopathic effects in resistant cells. Kaplan and Melnick (1955) reported that capuchin monkey kidney cells failed to adsorb significant amounts of poliovirus, and that the limited multiplication of type 1 poliovirus in such cultures, which showed no cytopathic changes, was explained by the fact that only one in 10,000 cells became infected. The situation with Coxsackie A9 and echo type 1 in patas cells was different in that these viruses adsorbed as well on patas as on rhesus; but it was similar in that neither virus produced plaques under agar nor cytopathic changes in fluid medium when the concentration of virus in the inoculum was low. However, when higher concentration of the same virus sample was used, irregular and minimal cytopathic changes were observed, and (in some but not all lots of patas cultures) tiny plaques developed. Not only were their numbers less (the efficiency of plating was about 10^{-5}, in comparison with plating on rhesus cultures), but the plaques grew at very slow rates and rarely exceeded 1 or 2 mm. in diameter (Figs. 1 and 2). Experiments were designed to determine the extent to which multiplication of these viruses took place.

Determination of growth curves of Coxsackie virus A9 in rhesus and patas cells. (*A*) Large inoculum: Coxsackie A9 containing 5×10^6 rhesus PFU in 0.1 ml. was introduced into each of 20 tubes of rhesus and 20 tubes of patas, from which the nutrient medium had been previously removed. All the tubes were incubated at 37° C. for 1 hour. At the end of the adsorption period, the supernatant fluid was removed from each tube and the cell sheet was washed five times with 1 ml. of PBS. Then 1 ml. of nutrient medium (0.5 per cent lactalbumin hydrolysate—2 per cent calf serum in Earle's salt solution) was added to each tube and the tubes were incubated at 37° C. At hourly and then daily intervals one rhesus tube and one patas tube were removed from the incubator, observed microscopically for cytopathic change, and frozen immediately at —20° C. The virus fluids were then titrated in rhesus bottle cultures. Virus titers were calculated in terms of PFU per ml. After a large inoculum of 5 million virus particles into a culture of 100,000 cells, cytopathic changes were

FIG. 1. Comparison of plaques produced by Coxsackie A9 virus 7 days after seeding in rhesus and in patas cultures.
Two bottles at left, Coxsackie A9 on rhesus: Big, circular plaques with hazy boundaries at inoculum of 0.1 ml. at 10^{-7}, 10^{-8}.
Two bottles at right, Coxsackie A9 on patas: Tiny irregular plaques at inoculum of 0.1 ml. at 10^{-1}, 10^{-2}. No plaques at lower concentrations of virus.

$$\text{Efficiency of plating} = \frac{\text{PFU per ml. plated on patas}}{\text{PFU per ml. plated on rhesus}}$$

$$\text{In this case: } \frac{1.3 \times 10^{4}}{2.7 \times 10^{9}} = 0.5 \times 10^{-5}$$

present in rhesus tubes at the end of 5–6 hours, whereas in the patas tubes such changes even after 48 hours were questionable. The growth curves in rhesus and patas cultures, and the development of cytopathic changes, are shown in figure 3. Although no definite cytopathic effect was observed in patas cells, the Coxsackie A9 did multiply to a limited extent, the yield being 10 per cent of that in rhesus cells. As with Coxsackie, under similar conditions, echo-1 multiplied in the patas cells, but again the yield was low, about 1 per cent of the yield in rhesus cultures.

With a small inoculum of nine virus particles, definite cytopathic effects were not observed in rhesus cells until 2 days after inoculation but as shown in figure 4, the virus had already multiplied to significant levels at the end of 8 hours. There was no

indication of virus multiplication or cytopathic effects of Coxsackie A9 in patas cells after as long as 14 days of incubation.

Thus neither echo-1 nor Coxsackie A9 virus produced plaques in patas cells under agar nor cytopathic changes in fluid medium when the virus inoculum was used in low concentration. However, both viruses grew in patas cultures to a limited degree when the inocula were large, but the cytopathic changes were questionable or minimal. Although the virus yield in patas cells was less than in rhesus, the shapes of the growth curves were similar in both monkey species. Minimal or questionable cytopathic changes occurred, and in certain lots of patas cells tiny plaques were observed, when high concentrations of the same virus samples were used. Figures 1 and 2 indicate that Coxsackie A9 and echo-1 produced clear plaques on rhesus cells at high dilu-

FIG. 2. Comparison of plaques produced by echo-1 virus 7 days after seeding in rhesus and in patas cultures.

Bottle at left, echo-1 on rhesus: Small, clear plaques with irregular boundaries at inoculum of 0.1 ml. at 10^{-6}.

Three bottles at right, echo-1 on patas: Tiny, irregular plaques at inoculum of 0.1 ml. of undiluted TC fluid and at 10^{-1} and 10^{-2}. No plaques at lower concentrations of virus.

Efficiency of plating in this case (see Legend for Fig. 1):

$$\frac{3.8 \times 10^4}{3.3 \times 10^8} = 1.1 \times 10^{-4}$$

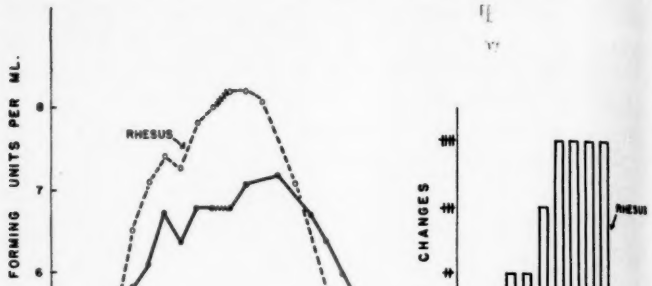


FIG. 3. Growth curve of virus and graph of CPE with large inoculum of Coxsackie A9 in tube cultures of rhesus and of patas cells. The CPE in patas monolayers was indefinite, as indicated by the plus-minus value.

Cytopathic changes:
± = Questionable
+ = ¼ of cells destroyed
++ = ½ of cells destroyed
+++ = ¾ of cells destroyed
++++ = Total destruction of cell sheet

tions of virus samples, but no plaques and no multiplication were observed when such high dilutions were inoculated on patas cells. However, tiny, delayed plaques appeared in cultures of a few patas monkeys when the inocula contained 10^4 to 10^5 or more rhesus PFU but in most patas monkeys plaques failed to appear even with inocula of 10^7 rhesus PFU. This phenomenon resembles the physiological restriction of certain attenuated strains of poliovirus in which, with large inocula, tiny delayed plaques appear at low concentrations of bicarbonate, whereas large, clear plaques appear at high concentrations of bicarbonate. The latter phenomenon has been described by Vogt and Dulbecco (1956), and has been confirmed in our laboratory using bottle cultures.

The adsorption experiments in this study showed that all the enteric viruses tested were able to adsorb on both rhesus and

patas cells regardless of the degree of viral susceptibility. Patas cells have a chromosome count of 54 and rhesus cells of 42 (E. H.-Y. Chu, unpublished data). The distinct genetic difference of these two species of host cells may furnish factors which either enhance the plaque-forming units of poliovirus or inhibit multiplication of Coxsackie A and echo A viruses.

That genetic factors may be responsible for inhibiting viral multiplication was found by Sabin (1952) in his study of the 17-D strain of yellow fever and other group B ARBOR viruses in PRI mice. Although 17-D virus multiplied in the brains of the resistant PRI mice, it produced no disease in them, this is similar to the lack of cytopathogenicity of the virus for patas cells which supported the limited multiplication of echo-1 and Coxsackie A9 viruses. The peak titers in the resistant mice were only 0.001 per cent of those achieved in the brains of susceptible Swiss mice; and the virus yields of Coxsackie A9 and echo-1 in patas cells were only 1 per cent to 10 per cent of those in rhesus cells.

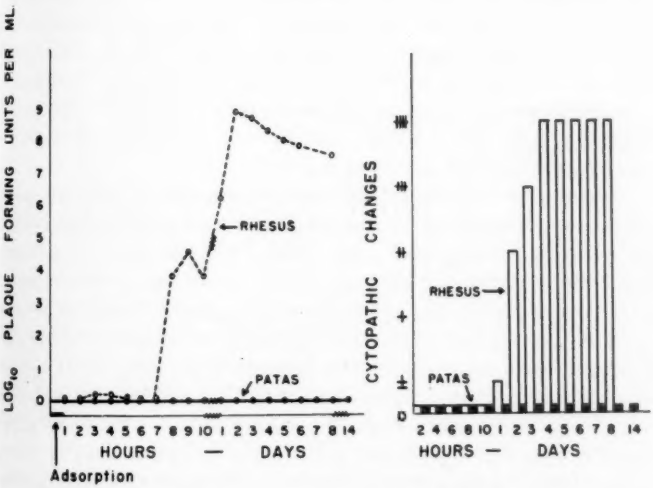


FIG. 4. Growth curve of virus and graph of CPE with small inoculum of Coxsackie A9 in tube cultures of rhesus and of patas cells. In patas cultures, no multiplication and no CPE was observed.
Legend as indicated for figure 3.

Maturation and Release of Virus from Cells

Until recently the animal virus of choice for studies in this field has been influenza. Now that an accurate assay is available for poliovirus, the inter-relationships of this virus and its host cell have become far more amenable to critical study, as witness the elegant study by Lwoff *et al.* (1955) on infection of single cells.

Amongst the prerequisites for studies correlating virus growth with biochemical or cytological changes is the strict definition of the kinetics of virus growth within, and release from, infected cells under various conditions of cell growth and manipulation. Such studies in most cases provide unequivocal results only when the conditions of the one-step growth experiment, first defined for bacteriophage by Ellis and Delbruck (1939), are satisfied. However, because animal cells tend to form aggregates or monolayers with intimate contact between cells, attempts to duplicate such ideal conditions by separating the cells may well be only partially successful due to distortion of the pattern of virus development and release as a result of damage to these cells.

Studies carried out with Howes, of the growth of a Type 1 poliovirus strain (Brunhilde) in monkey kidney cells, illustrate the pattern of virus development in this system and the necessity for treating with caution the correlations drawn between a given change in the behavior of the cell and the appearance of virus in the external environment of the cell.

Infection of cell suspensions and monolayers. Infection of a high proportion of cells was attempted by use of high input multiplicities of from 10 to 30 virus particles (PFU) per cell, and an adsorption period of 1 hour at 37° C. At the end of the adsorption period, the 0.5 ml. inoculum was removed and the plates washed five times with 5 ml. cold phosphate buffered saline. At this stage, to those plates for use in monolayer experiments, 3 ml. of growth medium at 37° C. were added. To obtain suspensions of infected cells, 2 ml. of dilute trypsin solution were added to one or two infected monolayers and the plates incubated at 37° C. for 10 to 15 minutes. The cell suspension was then lightly centrifuged, washed with PBS, recentrifuged, and finally suspended in growth medium at 37°. For growth curve experiments, the suspension was diluted to 50,000 cells per ml. and

transferred to an Erlenmeyer flask with indented sides which was placed on a magnetic stirrer. The cells were kept in suspension by gentle stirring with a suspended magnet.

Methods of harvesting extracellular virus and cell-associated or "intracellular" virus.

(a) Monolayers. At each time interval the supernatant fluid from one plate was removed. Those supernatants removed 5 hours or more after infection were centrifuged at 1,000 r.p.m. for 10 minutes in the horizontal head of an International refrigerated centrifuge to remove any infected cells which may have left the glass as a result of infection. To the cell monolayer, 3 ml. of freshly iced growth medium were added and the cells scraped off the glass by use of a rubber policeman. These "intracellular" and cell-free "extracellular" fractions were then stored frozen at —10° C. until further treatment prior to assay.

(b) Suspended cells. At each time interval 1 ml. of the infected cell suspension was removed and diluted tenfold with iced growth medium. The diluted cell suspension was then centrifuged at 1,000 r.p.m. as above, and 3–5 ml. of the supernatant fluid removed to represent the "extracellular" fraction. All but 1 ml. of the remaining supernate was then removed and discarded, and the 9 ml. removed were replaced with 9 ml. of freshly iced medium. This "intracellular" fraction was then stored frozen. In this way the risk of contamination of "extracellular" virus with infected cells was minimized, and contamination of intracellular virus with extracellular virus was held at a very small proportion of the intracellular.

Disruption of cells to obtain "intracellular" virus. Sonic vibration at 9,000 cycles per second (cps) was employed in the majority of experiments. This method produced breakdown of over 99 per cent of cells within 1 minute. Nuclei were disrupted more slowly than the whole cell, although even this fraction was reduced to less than 1 per cent of the original cell count within 3 minutes, the treatment accorded samples in these experiments, after which the cellular debris was very finely dispersed. Treatment with sonic vibrations at approximately 9,000 cps failed to result in any significant reduction of the plaque count of poliovirus.

In order to determine the proportion of cells in a suspension which produced virus, assays of such cell suspensions were made

using the plaque technique, within two hours of inoculation (*i.e.*, within the latent period of the virus). 0.3 ml. of each of the appropriate dilutions of cell suspensions were added to each of a series of plates which were then incubated at 37° C. for 30 minutes. Since attachment of the suspended cells to the monolayer appeared unlikely, 1 ml. of overlay was added to the plate and allowed to solidify before addition of the remaining 4 ml. of overlay. In this way it was hoped to avoid underestimation of the number of plaque-forming cells, by including cells of low yield which might fail to produce a plaque if held at too great a distance from the monolayer. Free virus was assayed in the same manner by use of the cell-free supernate of an aliquot of the suspension which had been centrifuged at 1,000 r.p.m. for 10 minutes.

Average multiplicity of infection. No attempt was made to determine this by assay of virus-producing cells put in suspension from the infected monolayer by means of trypsin, as this would probably have resulted in the death of a considerable proportion of the cells with consequent underestimation of multiplicity (when infected cell count was related to the original number of cells). Adsorption of less than 30 per cent of inoculated virus during the adsorption period had been observed on several occasions, and the plaque assay method was not considered accurate enough to afford reliable estimates of the small differences in concentration between the virus in the original inoculum and that remaining after adsorption. An indirect method for estimating the minimum value for multiplicity was therefore used.

Microscopic examination was made of a series of fixed and stained preparations of infected monolayers fixed at intervals of ½ to 1 hour during the growth cycle and removed from the glass by means of a collodion technique (Enders and Peebles, 1954; Reissig *et al.*, 1956). As shown in figure 5, this provided an estimate of the proportion of cells in which changes characteristic of virus infection occurred. Assuming the Poisson distribution to be applicable to the adsorption of virus to cells, and assuming the monolayer to be homogeneous with respect to the susceptibility of its cells, then from the proportion of uninfected cells the effective multiplicity may be calculated: per cent infected = $100(1-e^{-m})$, where m = average multiplicity of infection. In different experiments, between 62 and 91 per cent of cells mani-

fested changes characteristic of infection within 8 hours of inoculation. After 8 hours the percentage of cells with characteristic signs of infection fell to some extent, due to many infected cells rounding up and coming loose from the glass. Since no attempt was made to count the number of uninfected cells remaining per unit area of the glass surface, the maximum percentage of altered cells observed allows us to say that the effective multiplicity is no less than and probably more than that corresponding to the maximum percentage of altered cells observed. The minimum effective multiplicity therefore ranged from 1.0 to 2.4 compared to the actual input multiplicity of from 10 to 30.

In monkey kidney cells suspended by means of trypsin, the amounts of cell-associated or intracellular virus present during the period of virus increase were almost three times greater than the amounts of extracellular virus present at the same time inter-

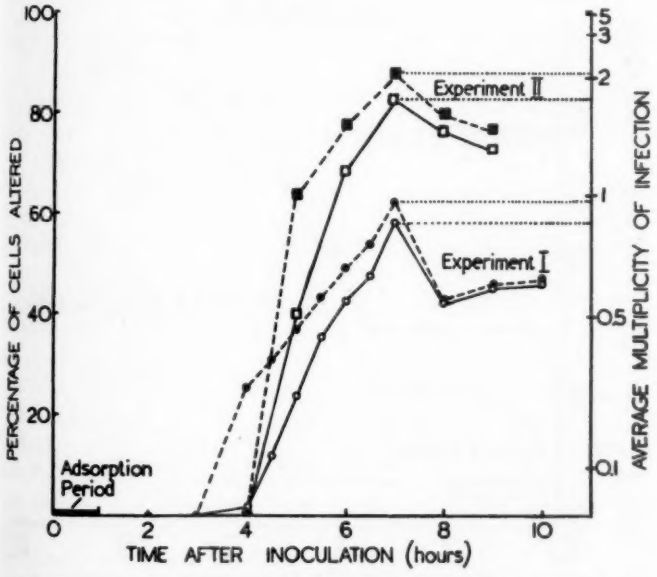


FIG. 5. Proportion of cells with virus-induced changes, with regard to time after inoculation of poliovirus type 1 (Brunhilde strain).
Broken line: Altered cells, all changes.
Solid line: Altered cells, excluding those with intranuclear inclusions only. (See Reissig *et al.*, 1956).

vals, the two curves being parallel during this period (Fig. 6). While this difference may at first sight appear small, it indicates the existence of an appreciable time lag between maturation of a virus particle and its release from the cell. The "average re-

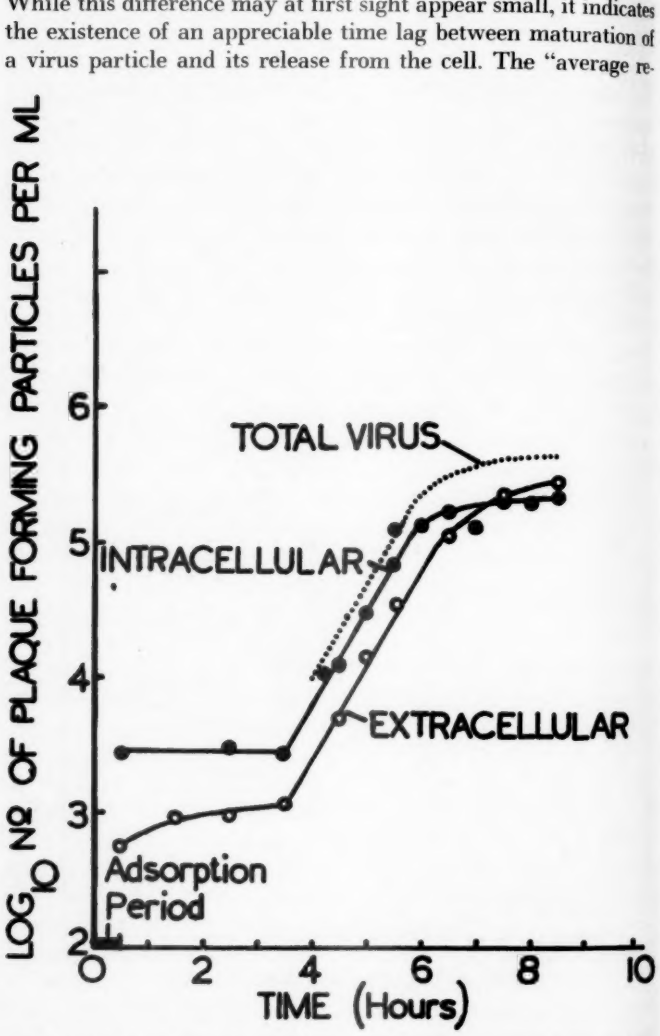


FIG. 6. One-step growth curve of type 1 poliovirus (Brunhilde strain) in a suspension of monkey kidney cells. Total virus obtained by adding the amounts of intra- and extracellular virus found at each time of observation.

lease time" of this virus strain from suspended cells was approximately 90 minutes, that is, the time between the maturation of the virus particle within the cell and its appearance outside the cell.

The data in table 5 were obtained with second passage monkey kidney cells suspended by means of versene and infected, while in suspension, with type 1 poliovirus. Assays of the number of plaque-forming cells were made within two hours of infection and of the virus yield at 22.5 hours after infection. The relationships between the total cell count, the count of cells capable of producing plaques when plated on monolayer cultures, and the virus yield of the infected cell suspension indicate that, even under conditions in which 99 per cent of cells adsorbed virus, only 17.6 per cent appeared capable of producing virus. Although

TABLE 5

Comparison of Data on Cell Counts, Virus Adsorption, and Virus Yield for a Suspension of Monkey Kidney Cells Prepared with Versene and Infected with Type 1 Poliovirus

I. *Hemocytometer counts**	
Total cells	1.5×10^6 per ml.
Undamaged cells†	1.0×10^6 per ml.
II. *Adsorption data*	
Initial virus concentration	5.6×10^6 PFU per ml.
Concentration remaining after 30 minutes adsorption at 37° C.	0.5×10^6 PFU per ml.
Virus adsorbed	5.1×10^6 PFU per ml.
Average multiplicity	5.1 PFU per cell
III. *Proportion of undamaged cells infected* (from I and II and assuming Poisson distribution)	99.4 per cent
Concentration of infected cells	9.9×10^5 per ml.
IV. *Counts of plaque-forming cells*	
Infected cells plus free virus	2.08×10^5 plaques per ml.
Free virus alone	0.32×10^5 plaques per ml.
Infected cells	1.76×10^5 plaques per ml.
Therefore, $\frac{1.76 \times 10^5}{9.9 \times 10^5} = 17.6$ per cent of cells produce virus	
V. *Virus yield* (Cell suspension diluted tenfold)	
Virus concentration after 22.5 hrs. incubation	1.44×10^6 PFU per ml.
Cell concentration (from undamaged cell count)	10^5 cells per ml.
Yield per cell on basis of hemocytometer count	14.4 PFU
Yield per cell on basis of count of plaque-forming cells	82 PFU

* Cells stained with 0.05 per cent crystal violet in 0.05 per cent citric acid.
† Cells which showed a complete and apparently undamaged cell membrane.

use of monolayers in place of cell suspensions is undesirable, because of the possible adsorption of released virus and consequent distortion of the curve of extracellular virus, the probable but not readily assessable damage to cell during removal from the monolayer may produce even grosser distortion of the kinetics of virus release from infected cells than could be produced by adsorption of released virus. With this in mind, the growth of virus in monolayers of monkey kidney cells was followed, the cells being removed from the plates only at the time of harvest of virus.

The curves for intra- and extracellular virus increase in cells of infected monolayers showed even more marked quantitative differences than had been observed for suspended cells. The increase in concentration of the intracellular virus observed was detected within three hours (or less) of the initial detection of new virus at this site, and maturation was virtually complete within a further two hours. In the two experiments represented in figure 7, the experimental manipulations were as nearly identical as possible, except that the minimum average multiplicities of infection attained were approximately 0.8 and 1.8 for experiments I and II, respectively. A substantial difference was observed between times at which virus release could first be detected in the two experiments, and this might have resulted from the greater heterogeneity of response of the cell population used in Experiment I. This possibility was suggested by Dr. Reissig's cytological observations on parallel series of plates (Fig. 5), and indicates on the average a longer "latent period" for the cells used in the first experiment than those used in the second.

Over the 11-hour period of observation in Experiment I (Fig. 7), only 19 per cent of the maximum virus yield had appeared in free form outside the cell. At the end of this period approximately 50 per cent was still attached to or within the infected cells remaining fixed to the glass, while a further undetermined amount was present in cells which had come free from the glass. This fraction was discarded with the cellular deposit from the centrifugation of the fluid phase to obtain free virus. If we assume that the cells in this experiment had produced their maximal amount of virus by the seventh hour, then the amount of virus irreversibly absorbed to cells must have been considerably less than 30 per cent. We feel therefore that absorption is unlikely to have been sufficient to account for the wide discrepancy

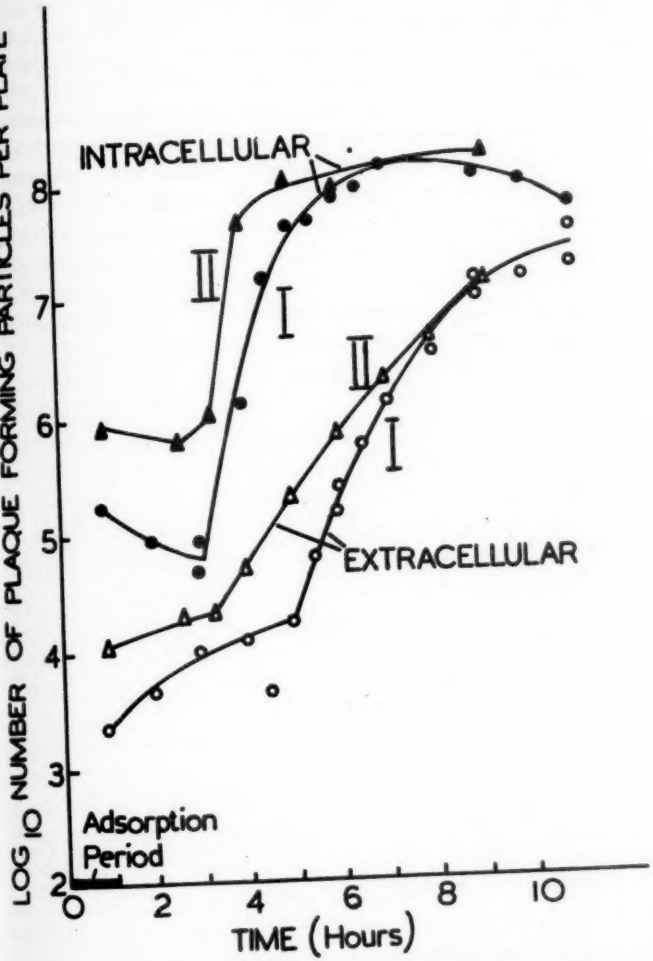


FIG. 7. One-step growth curves of type 1 (Brunhilde strain) poliovirus in monkey kidney monolayers, in two parallel experiments, differing only in average multiplicities of infection, which were approximately 0.8 and 1.8 for Experiments I and II, respectively.

between intra- and extracellular virus concentrations observed during the early stages of the growth cycle.

For the Brunhilde strain used in our experiments, the "average maturation time" (the time between inoculation and the appearance within the cells of 50 per cent of the total infective virus produced) was estimated at approximately 6 hours for suspended cells (Fig. 6), and between 5 and 6 hours for monolayers (Fig. 7). The high value of 90 minutes obtained for the "average release time" (time between the maturation of intracellular virus and its extracellular appearance) of type 1 poliovirus from monkey kidney cells in suspension, and the still higher value which may be inferred but not calculated for cells in monolayers, is in marked contrast to that obtained for the western equine encephalomyelitis (WEE) virus—chick fibroblast system by Rubin *et al.* (1955) for which an "average release time" of one minute or less was found.

To account for the wide discrepancy found between the amounts of intra-and extracellular virus during the earlier portions of the growth cycle in cell monolayers, and the marked difference in *rates of increase* of these virus fractions, Howes has advanced two explanations, differing in the main only in degree:

1. The process of virus release may follow immediately upon the appearance of the first mature virus particles within the cell. Release of virus from the cell may continue for relatively long periods, although the amount released per unit time may be limited. Should virus mature more rapidly than it can be released, mature virus will accumulate within the cell, and intracellular and extracellular virus concentrations will not bear a constant relationship to one another. The curves for increase in intra- and extracellular virus will therefore be divergent, as was observed in infected monolayers. This explanation might be expressed briefly as continuous but rate-restricted release.

2. Once the release process begins, it may be rapid, yet mature virus may be held within the cell for a relatively long period before release is initiated. A cell might conceivably continue to hold mature virus within it even after the processes of virus synthesis and maturation have ceased. In this case also, the rates

of release and maturation would be independent, and would agree with the experimental findings.*

Whatever interpretation is placed on the data obtained from the experiments described in this paper, they suggest a possible fundamental difference between the growth cycle of poliovirus in monkey kidney cells, and that of such viruses as WEE in chick fibroblasts where release is rapid and the potential rate of release greater than the rate of maturation (Rubin *et al.*, 1955). This difference might lie in a physico-chemical barrier to release associated with a structure normally present in the cell but perhaps modified by infection, or with abnormal structures present only in the infected cell (Reissig *et al.*, 1956). It is noteworthy that infection of the monkey kidney cell is associated with the appearance of intranuclear inclusions, an eosinophilic mass in the perinuclear area of the cytoplasm, and—in the late stages of infection—with vacuolization of the cytoplasm. It is equally possible that virus release is initially retarded by the intact cell membrane, and that free egress of virus from the cell follows damage of the membrane at a late stage in the growth cycle of virus, and as a consequence of infection.

The results obtained in this study are also of value in view of the increasing use of tissue culture in quantitative studies, for the differences between growth curves in suspended cells and in cell monolayers may well be due largely to the damage incurred by cells during suspending procedures. The fact that at present the amount of damage is not known and is difficult to evaluate, indicates the need for caution in assessing results obtained with

* Lwoff *et al.* (1955), by examination of the virus yields of single cells in microdrops, concluded that release of the major part of poliovirus from the cell occurs within a period of 30 minutes. They suggested that release accompanies partial lysis of the cell. They also noted that the cells examined showed a considerably longer latent period than that observed in mass cultures, an observation consistent with rapid release from individual cells. Their data conform more closely to the second alternative proposed above regarding the nature of release. With rapid rather than gradual release from single cells, the extracellular virus curve obtained with mass cultures would reflect the statistics of the population heterogeneity rather than the kinetics of the process of virus release itself. This may be followed reliably only by studying release from large numbers of single cells under carefully controlled conditions. Under these circumstances, the first part of the curve of extracellular virus increase (involving virus increase over a range of several logs) might be determined by a small proportion of cells which release virus early.

suspended cell systems, for the degree to which cells are damaged may vary to a marked extent with apparently minor variations in technique.

The results have further bearing on studies aimed at correlating the biochemical activities of the cell, or morphological change, with virus production. With virus-cell systems in which virus release is rapid, and its initiation in no way delayed, it is probably sufficient to use the extracellular virus concentration as a simple means of following virus synthesis. With the poliovirus-monkey kidney cell system, and perhaps other systems, extracellular virus assays are insufficient, for maturation is virtually complete several hours before even half of the virus is released from the cells, and changes associated with dissolution of the cell may well be linked erroneously with virus synthesis.

Studies on Poliovirus Synthesis in Monkey Kidney Cells Grown in Synthetic Medium

Within the last two years a synthetic medium for the growth of monkey kidney cells has been developed in our laboratory by Dr. Catherine Rappaport (1956–1957). The effect of some specific agents on the growth of the cell and on the growth of poliovirus have been studied and will be discussed here.

The synthetic medium, SM-2, is shown in table 6. It contains the essential amino acids, and also cysteine, glutamine, and glycine. Both pyruvate and glucose are used as carbon sources. There is a supplement of the water soluble vitamins and also lecithin and cholesterol. The basic salt solution is essentially that of Hanks supplemented with additional calcium and trace elements.

It is not known how many of the constituents in SM-2 are required. It has not been possible to demonstrate a requirement for most of the vitamins within the first three weeks of *in vitro* life. The medium SM-2 has been found to support at least 4–5 divisions of the cells and a variable lifetime of 3–6 weeks (see Fig. 8). Cultures maintained in SM-2 have in every case survived as long as, or longer than, parallel cultures in the complex 2 per cent calf serum-lactalbumin hydrolysate medium. The addition of serum to SM-2 does not increase either the rate or duration of cell growth. Glycine is required for even one division

TABLE 6

Synthetic Medium (SM-2) for Growth of Monkey Kidney Cells

Amino Acids	mg./liter	Vitamin Mixture	mg./liter	Basic Salt	g./liter
l-isoleucine	300	Folic acid	40.0	NaCl	8.0
l-leucine	200	Biotin	0.15	KCl	0.4
l-lysine	60	Nicotine acid	0.15	$MgCl_2 \cdot 6\,H_2O$	0.1
l-histidine · HCl	10	Pyridoxine	0.115	$MgSO_4 \cdot 7\,H_2O$	0.1
l-valine	170	Thiamin	0.15	Na_2HPO_4	0.06
l-arginine	60	Pantothenate	0.10	NaH_2PO_4	0.06
l-phenylalanine	90	Riboflavin	0.15	$CaCl_2$	0.4
l-tyrosine	145	Vit. B_{12}	0.015	$NaHCO_3$	See note
l-tryptophan	5	Choline	1.00		
l-methionine	12	Inositol	3.50	Trace Elements	M x 10^{-6}/liter
l-threonine	60	Ascorbic acid	50.00	$Fe(NO_3)_3$	2
l-cysteine · HCl	100			$CoCl_2 \cdot 6\,H_2O$	0.2
glycine	150	Miscellaneous	mg./liter	$MnCl_2 \cdot 4\,H_2O$	0.5
l-glutamine	280	Lecithin	25	$ZnSO_4 \cdot 7\,H_2O$	3
		Cholesterol	10	$(NH_4)_2MO_4$	1
		Ethyl alcohol (a diluent for lecithin and cholesterol)	590	$CuSO_4 \cdot 5\,H_2O$	0.4
		Glucose	1000		
		Pyruvate (sodium salt)	500		

Notes on preparation:

Amino Acids—Held as 20 x concentrated combined stock without tyrosine, cysteine, glutamine. Tyrosine held as 0.2 per cent stock; cysteine held as 10 per cent stock at 4° C.; glutamine held as 2 per cent stock at —20° C. Amino acids sterilized by filtration.

Vitamin Mixture—Held as 100 x conc. stock without ascorbic acid. Sterilized by filtration.

Miscellaneous—Lecithin (Pfanstiehl ex ova) held as 10 per cent stock in absolute ethanol; cholesterol as 2 per cent stock in absolute ethyl alcohol.

Basic Salt—Held as Hanks salt 10 x conc. with glucose; supplemented with 0.26 gms./liter of $CaCl_2$, and with bicarbonate, after the medium has been made up. $CaCl_2$ held as 10 per cent stock. $NaHCO_3$ concentration for outgrowth from trypsinized suspensions, 1.1 gms./liter; for replenishment, 1.6 gms./liter.

Trace Elements—Held as separate stocks 0.05 per cent conc. $Fe(NO_3)_3$ sterilized by filtration.

To prepare 1 liter—Add in order to 750 ml. distilled demineralized H_2O:

		ml.
Hanks 10 x conc. with glucose		100
SM-2 Amino Acid conc. 20 x		50
Tyrosine 0.2 per cent heated to dissolve		70
Glutamine 2 per cent		14
Cysteine 10 per cent		1
Vitamin Mixture 100 x conc.		10
Ascorbic Acid 10%		0.5
Lecithin		0.25
Cholesterol		0.5
$NaHCO_3$ (7.5% solution)		15–25 ml. (see note above)
$CaCl_2$	10%	2.6
$ZnSO_4 \cdot 7\,H_2O$	.05%	0.2
$Fe(NO_3)_3 \cdot 9\,H_2O$	.04%	0.2
$CuSO_4 \cdot 5\,H_2O$	.05%	0.05
$CoCl_2 \cdot 6\,H_2O$	.01%	0.05
$MnCl_2$	.01%	0.10
$(NH_4)_2MoO_4$	.05%	.2

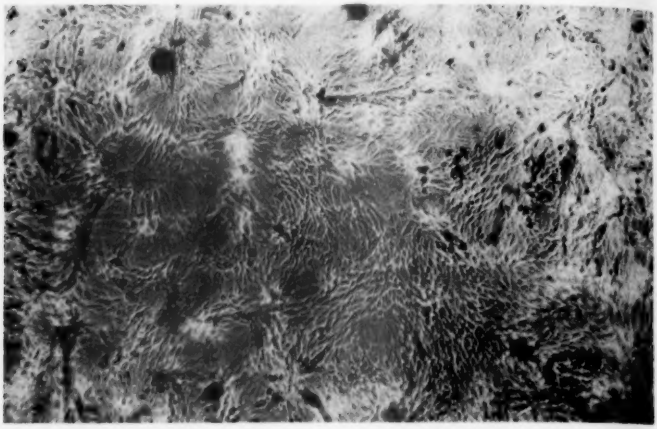
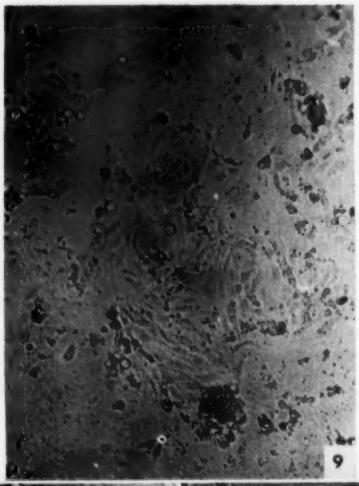


FIG. 8. Three week old culture of monkey kidney cells grown in synthetic medium SM-2 in alkali-rinsed Pyrex 7740 flasks. Low magnification.

by four-day-old cultures. To demonstrate this, cells were grown out in 2 oz. flat prescription bottles in the synthetic medium SM-1. After 4 days the medium was decanted, the cultures washed 2 times with Hanks salt solution and transferred to SM-2 with and without glycine. In the absence of glycine the population remained essentially constant but at 1.8×10^{-3} M glycine there was a continuous increase in the cell number until the growth had completely covered, and was limited by the glass surface available. It has not been possible so far to replace glycine with nucleic acid derivatives.

In a similar manner it has been found that a concentration of calcium 3–4 times higher than that in Hanks original formula is necessary for growth. Without this calcium supplement, the cells stop spreading along the glass, the edges of the cells become thickened and the cells granulated (see Figs. 9, 10, and 11).

Even more critical than the concentration of calcium or the presence of glycine is the type and conditioning of the glass used for the cultures. The medium SM-2 was developed using the relatively soft glass in the Brockway prescription bottles, or Corning pyrex No. 9800. The importance of the type of glass and thus the demonstration of the exquisite sensitivity of these cells to physical factors is shown by the absolute requirement for a dilute



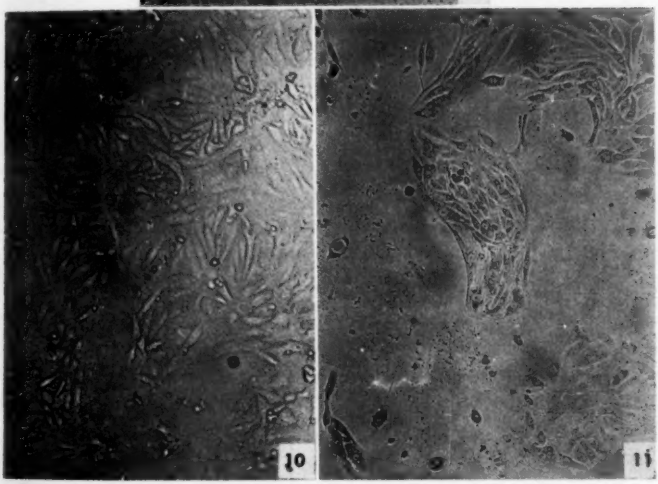


FIG. 9. Culture of monkey kidney cells grown out in SM-2 for three days (before replenishment of medium). Shown for comparison wth figures 10 and 11.

FIG. 10. Culture from the same series as that in figure 9, shown 24 hours after change to SM-2 with recommended high calcium concentration (0.4 gms./liter).

FIG. 11. Culture from the same series as those in figures 9 and 10, shown 24 hours after change to SM-2 with low calcium concentration (0.2 gms./liter).

NaOH (0.2 N) rinse when using Corning pyrex glass No. 7740. With pyrex glass No. 7900, a glass with even lower cationic exchange properties than No. 7740, no treatment has been found which will permit growth.

It is not possible here to mention other observations on the effect of physical factors on the quality of cell growth in the absence of protein. The observations are consistent with the hypothesis that the negatively charged cell is attached to the negatively charged glass through a divalent cation. The surface bound cation is easily exchanged for the hydrogen ion produced by the cells during metabolism with a consequent inhibition of attachment and spreading of the cells along the glass. If this is the case the growth of the cell in a synthetic medium would be controlled by the concentration of cations in the medium as well as by cationic exchange capacity of the glass. These relations would be expected to be less critical in the presence of protein bound to the cell surface and to the glass.

Cells grown and maintained in the medium SM-2 have been studied for susceptibility to poliovirus (Mahoney, type 1), and compared to cultures grown in the 2 per cent calf serum-lactalbumin hydrolysate, or "M" medium. It has been found that poliovirus is propagated in either M or SM-2 monolayer cultures in a salt-glucose medium in the absence of any exogenous nitrogen source. Typical propagation data are shown in table 7. It is seen that the majority of cultures synthesize about 200 plaque-forming units per cell—a value found by Lwoff *et al.* (1955), and

TABLE 7

Virus Propagation in Hanks Salt-Glucose Solution

Exp. No.	Av. No Cells Per Culture ($\times 10^6$)	PFU (Log.) Per Culture at Hours				PFU Per Cell
		16–24	40–48	55–60	69–80	
1	2.8	...	6.40	8.28	9.04	360
2	2.8	...	7.10	...	8.85	250
3	2.8	...	8.95	...	...	322
4	1.8	4.85	7.85	7.90	...	44
5	2.8	6.80	8.85	...	...	250
6	2.8	...	6.55	7.60	...	14
7	2.8	...	7.14	9.04	...	360

M cultures in bottles each inoculated with an average of 5 to 10 PFU, suspended in Hanks solution. After 1 hour at 37° C., cultures were washed three times with PBS and replenished with 10 ml. of Hanks salt solution with 1.0 mg./ml. of glucose.

by Youngner (1956) to be characteristic for infected monkey kidney cells in a complex nutrient medium.

It has been found that the rate and amount of virus produced during the first 24 hours after the cells are transferred to the salt solution is independent of an exogenous carbon source. The kinetics of virus synthesis in monolayer cultures of SM-2 cells is shown in figure 12. A series of cultures were inoculated with 3,000 PFU diluted in a glucose-free salt solution. After incubation at 37° C. for 1 hour, when about 10 per cent of the virus had adsorbed, the cultures were washed three times to remove any residual virus. They were incubated in a salt solution (Hanks), containing 0.9 grams of bicarbonate per liter, with or without glucose. The concentration of glucose used was 2.5 mg./ml., since other studies had shown that this would maintain a maximum rate of glucose uptake during a 24-hour period. At intervals, one bottle from each series was removed, and the virus in the medium was titrated. The monolayer was rinsed thoroughly to remove extracellular virus, then scraped and collected in a few ml. of salt solution. This preparation was frozen and thawed three times and then titrated for cell-associated virus.

The growth curves in figure 12 show that after adsorption there is a latent period characterized by a variable decline in virus which can be recovered in the monolayer. After about three hours there is a sharp rise of the virus in the cultures. The total virus bound to or within the cells is considerably greater than the virus free in the medium, consistent with the findings referred to earlier in this paper. In the present experiment it is seen that the entire sequence of events, including synthesis, release, re-adsorption and synthesis throughout the 24-hour period is the same in cultures metabolizing glucose at a maximum rate as in cultures in the absence of exogenous carbon.

Thus, the nitrogen, organic carbon, and all energy for the synthesis of virus must be derived from the endogenous reserves of the cell. Since the total endogenous polysaccharide of the culture of 10^6 cells varies between 30–50 gamma equivalents of glucose and since the cultures can utilize 200 gamma of glucose per hour, it would seem that the amount of energy used for synthesis of virus during the 24 hours is a very small fraction of the cell's total energy-producing capacity.

The propagation of virus after 24 hours has been found to re-

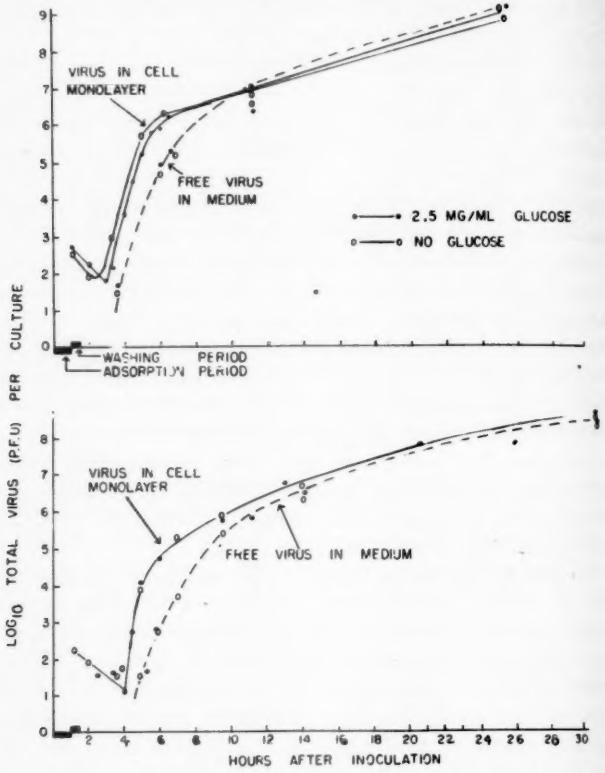


FIG. 12. Kinetics of poliovirus synthesis in monkey kidney cells in the presence and absence of glucose. Nine day old monolayer cultures of monkey kidney cells, washed and then inoculated with 3 x 10^3 plaque-forming units per culture. Virus suspended in Hanks salt solution with or without glucose. After 1 hr., cultures washed free of unadsorbed virus and replenished with Hanks salt solution. Intracellular (plus cell-bound) virus and free virus in the medium titrated by plaque technic at times indicated.

quire glucose, presumably due to the need for a carbon source for maintenance of the cells in a salt solution. Pyruvate, which is rapidly utilized by these cells, however, only partially replaces glucose for virus synthesis.

The different constituents of the medium SM-2 have been studied for their effect on virus propagation in a salt-glucose

medium. The only two constituents which have been found to affect the course of virus propagation are cystine and glycine.

Typical data for the effect of cysteine (cystine) in this system are given in table 8. It is seen that at any given glucose concentration, cysteine decreases the time when cytopathogenic changes

TABLE 8

Stimulatory Effect of Cysteine on Virus Synthesis in M Cultures in Salt-Glucose Solution

Medium: Hanks Salt Solution, Supplemented With:		Time of Harvesting (When CPE is Complete) (Hours)	PFU Per Culture (Log.)	Virus Yield Per Cell (PFU)
Carbon Source: Glucose (mg./ml.)	Nitrogen Source: Cysteine (μg./ml.)			
3.0	..	90	8.8	260
3.0	50	60	9.4	1050
0.9	..	49	9.0	400
0.9	50	43	9.4	1050
0.3	..	49	8.6	270
0.3	50	49	9.0	400
3.0	..	95	8.4	110
3.0	50	55	9.0	400
0.9	..	71	8.7	200
0.9	50	55	9.2	630
0.3	..	71	8.3	77
0.3	50	47	9.1	520

M cultures in bottles each inoculated with an average of 5 to 10 PFU, suspended in Hanks solution. After 1 hour at 37° C., cultures were washed three times with PBS and replenished with 10 ml. of Hanks salt solution supplemented as indicated.

are complete, and increases the yield of plaque-forming units per cell (Rappaport, 1956). Recently Dubes (1956) has presented data using other strains of poliovirus which confirm the stimulatory effect of cysteine on virus synthesis. The role of cysteine is not understood but it may be pointed out that in these experiments the glucose for maintenance and energy is constantly declining and thus the possibility that cysteine serves as a source of pyruvate has to be considered.

Regardless of the mechanism of the action of cysteine, it is seen that the yield of virus per cell may be as much as 1,000 after 48–60 hours of propagation. This value should be corrected for any inactivation of virus taking place in a slightly alkaline salt solution at 37° C. Studies on the stability of poliovirus under these conditions indicate that at least 50 per cent of the active

virus particles (plaque-forming units) are lost during the first 24 hours. Using a preparation of virus grown in medium 199 and stored in Hanks salt solution for 2 years at —20° C., 40 per cent and 60 per cent of the virus was recovered in two experiments. Using a virus preparation which was obtained by harvesting the monolayers and liberating the cellularly bound virus by freezing and thawing, only 5 per cent of the virus was recovered. This difference in the stability of old and new virus preparations makes it difficult to correct for the inactivation occurring during propagation. A correction factor of 2 probably represents a lower value. If the yields obtained in the presence of cysteine are corrected for inactivation it is seen that the monkey kidney cell can produce at least 2,000 active virus particles per cell.

As has already been presented, glycine is required for growth of monkey kidney cells. Glycine, however, inhibits the synthesis of poliovirus. Figure 13 shows the effect of glycine on virus propagation in the salt-glucose medium. It is seen that concentrations of glycine lower than that required by the cells for growth significantly inhibit the synthesis of virus. Studies of the first cycle of virus synthesis, where the glycine has been added after adsorption, indicate that 3×10^{-4} M glycine decreases the yield of virus about 90 per cent and prolongs the latent period by about one hour. Figure 14 shows that in the presence of glycine plaque numbers are reduced under a protein-free synthetic medium used

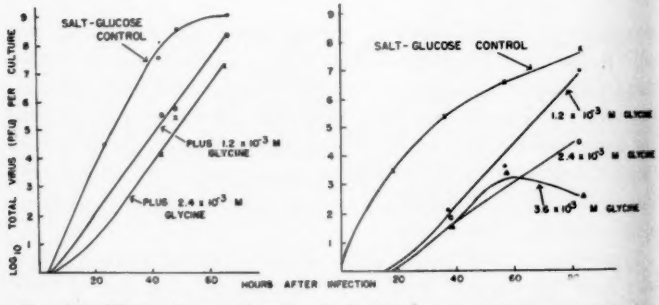

SALT-GLUCOSE CONTROL
PLUS 1.2 x 10⁻³ M GLYCINE
PLUS 2.4 x 10⁻³ M GLYCINE
LOG₁₀ TOTAL VIRUS (PFU) PER CULTURE
HOURS AFTER INFECTION
SALT-GLUCOSE CONTROL
1.2 x 10⁻³ M GLYCINE
2.4 x 10⁻³ M GLYCINE
3.6 x 10⁻³ M GLYCINE

FIG. 13. Effect of concentration of glycine on assay of type 1 poliovirus (Mahoney strain) using salt-glucose medium. Nine day old cultures grown in 3 oz. bottles with "M" medium were inoculated with 5-10 PFU. After 1 hr. at 37° C., cultures were washed 3 times with PBS, and replenished with Hanks salt solution with or without glycine. Two separate experiments are represented.

in the agar overlay (Rappaport, 1956), and the rate of growth of the plaques is retarded.

It was possible that the inhibition by glycine represented a non-specific toxicity due to the deranged metabolism of the cells in the presence of a single amino acid. In order to eliminate this possibility, the propagation of poliovirus was studied in cultures in the presence of the complete growth medium SM-2 and in SM-2 minus glycine. The results in figure 15 show that there was a 2 to 3 log inhibition of poliovirus synthesis during the first 40 hours in the medium containing glycine. A replenishment of the medium, and thus of the glycine, every ten hours prolonged this inhibition.

In concluding this section, it may be said that a synthetic medium has been developed which supports the outgrowth and proliferation of monkey kidney cells. Growth in this medium depends on defined chemical and physical conditions, including calcium and magnesium concentrations and the surface of the glassware. Glycine may stimulate growth as early as four days after the cells have been removed from the monkey. Poliovirus may be synthesized in the absence of any source of exogenous

FIG. 14. Plaque inhibition by presence of glycine in overlay at molarity indicated. C=control culture, in absence of glycine.

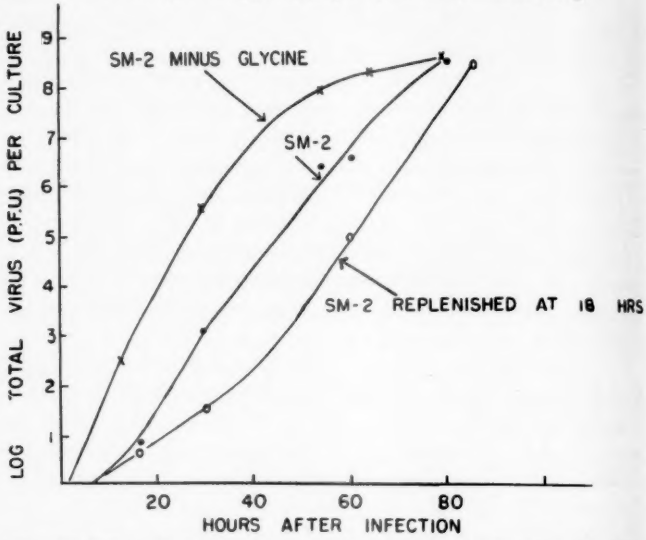


FIG. 15. Effect of glycine on propagation of type 1 poliovirus using complete growth medium SM-2, and SM-2 minus glycine. Ten day old 2 oz. bottle cultures grown in SM-2, were inoculated with 5–10 PFU. After 1 hr. at 37° C., cultures were washed and replenished with indicated medium. There was a 2 to 3 log inhibition of poliovirus synthesis during the first 40 hrs., in the medium containing glycine.

nitrogen, organic carbon, or energy. The synthesis of this virus thus represents efficient utilization of preformed constituents of the cell. Only two supplements have been found to affect significantly the propagation of poliovirus in this system. Cysteine, or cystine, stimulates the rate of propagation and increases the yield of virus per cell. Glycine which stimulates growth of the cell specifically inhibits the synthesis of the virus. It appears from these studies that the synthesis of poliovirus by monkey kidney cells is not an invariant consequence of the adsorption of the virus, but may be controlled by specific transients in metabolism. These transients, favorable and unfavorable to virus synthesis, may be induced by compounds which are physiological for the cell.

Effect of Cell Nutrient on Cytopathogenic Response

Effect of glucose uptake on cytopathogenic effect (CPE) of poliovirus. That poliovirus may be propagated in monkey kid-

ney cells independent of any exogenous carbon or nitrogen source for 24 hours has been demonstrated in the experiments discussed above (see Fig. 12). After 24 hours glucose is required, presumably because the endogenous polysaccharide, or other reserves of the cell, are depleted after 24 hours in a salt solution. In the course of these studies, Dr. Rappaport also noted that the cytopathogenic effect (CPE) of the virus was strongly influenced by the concentration of glucose, or rather by the glucose uptake. With a concentration of glucose of 0.3; 0.9; or 3.0 mg./ml. glucose uptake stopped after 15, 40, and 58 hours, respectively, and in each case, the CPE occurred shortly after the cessation of glucose uptake, regardless of the amount of virus which had been produced (see Fig. 16). The kinetics of virus synthesis are the same regardless of glucose concentration up to 24 hours. At that time CPE was already present in the cultures with no glucose, and virus production soon ceased in them. Virus propagation continued in the cultures containing glucose, but with CPE being delayed until glucose uptake ceased. There was a much better correlation between the time when glucose uptake stopped and CPE than there was between virus yield and CPE.

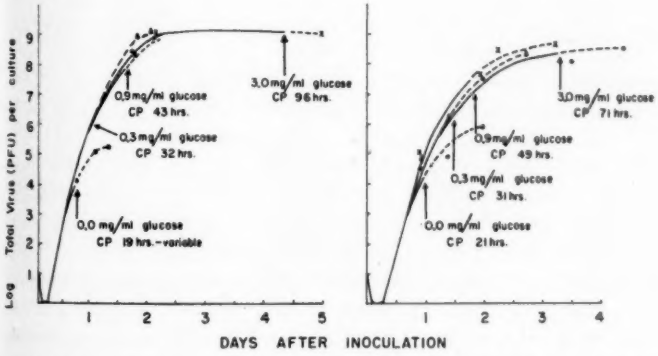


FIG. 16. Effect of concentration of glucose on cessation of CPE in monkey kidney cells infected with type 1 poliovirus. The time at which glucose uptake stopped in these experiments was 18 hrs. when the initial concentration was 0.3 mg. glucose per ml., 36–40 hrs. with 0.9 mg. glucose per ml., and 50–70 hrs. with 3.0 mg. glucose per ml.

Solid line = Virus production before CPE.

Broken line = Virus production after CPE.

Arrows = Time of first signifiacnt CPE, about 30 per cent of culture.

Effect of glutamine on CPE of measles virus. Another striking example of the effect of cell nutrient has recently been demonstrated by Reissig *et al.* (1956), whose results are illustrated in figures 17–24. With measles virus in a human epidermoid cell line (Hep-2), two completely different morphological patterns of degeneration were obtained depending on the composition of the nutrient medium employed for virus propagation.

Hep-2 cells were grown in Eagle basal synthetic medium supplemented with 10 per cent calf serum. Well developed cell sheets (Figs. 17 and 18) were inoculated with the Edmonston strain of measles virus. At the time of inoculation, the growth medium was replaced with either fresh Eagle medium containing 5 or 10 per cent calf serum, or with biological medium recommended by Enders, composed of 35 per cent bovine amniotic fluid, 5 per cent beef embryo extract, 25 per cent heated horse serum and 35 per cent Hanks balanced salt solution. Cytopathic changes appeared from 2 to 14 days after virus infection, depending on the dose of virus inoculated. When Enders medium was used as nutrient, flat syncytial masses appeared in the inoculated cultures, as described by Enders and Peebles (1954). Some of the giant cells contained several hundred nuclei (Fig. 19) and in the last stages the whole cell sheet, of over 1 cm^2, was transformed into a few huge syncitia (Fig. 20). In the cultures nourished in Eagle medium, the cells after infection become elongated and spindle-shaped, or rounded (Figs. 21 and 22). Very few polynucleated cells were present in each culture, and most of these contained less than ten nuclei each.

Evidence was obtained to show that a deficiency in the Enders medium was a responsible factor in the appearance of multinucleated cells. When Enders medium was supplemented with the amino acids, vitamins, and glutamine contained in Eagle medium, (or, at times, even when the supplement was glutamine alone) giant cell formation was greatly reduced, and most of the cells involved were typical of measles degeneration in Eagle medium alone. That the effect could be attributed primarily to glutamine was proven by its omission from the Eagle medium. With this deficient medium the appearance of infected cultures was very similar to that in Enders medium (Figs. 23 and 24). These results are summarized in table 9. When nourished by the media listed on the left, which contain excess glutamine, cells infected

with measles virus degenerate individually. When cells are nourished by the media listed on the right—which are deficient in glutamine—the virus-induced degeneration is characterized by an entirely different cytopathic response, in which giant multinuclated cells are the prominent feature.

Differences in the cytopathic effect related to nutrient medium were also observed with other viruses, namely with two strains

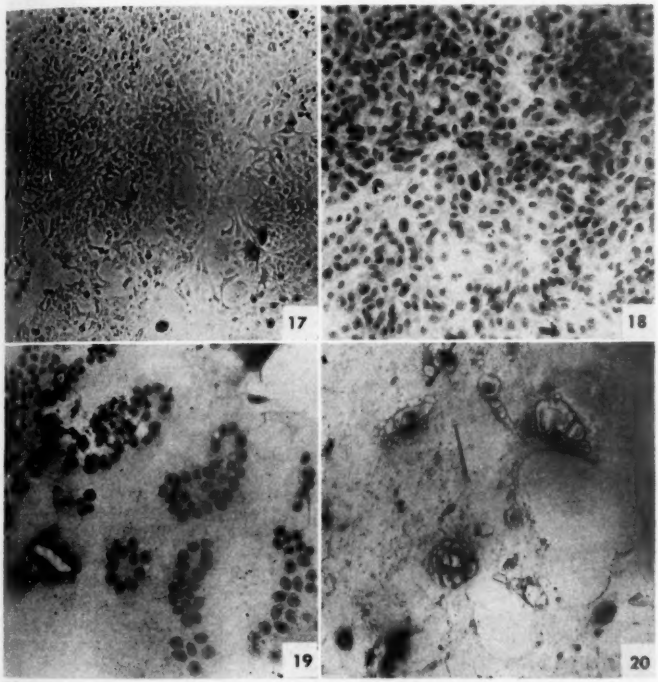


FIG. 17. Uninoculated control culture of Hep-2 cells maintained in Enders medium, photographed in the living state. About 50 X.

FIG. 18. Uninoculated control culture of Hep-2 cells maintained in complete Eagle medium. The cells are more densely packed than those maintained in Enders medium. Hematoxylin and eosin stain. About 120 X.

FIG. 19. Culture of Hep-2 cells maintained in Enders medium, in a late stage of measles virus-induced degeneration. The whole monolayer is transformed into a few syncytial masses, containing clusters of nuclei which share a common cytoplasm. Fixed and stained with hematoxylin and eosin. About 120 X.

FIG. 20. Culture similar to that shown in figure 19, but photographed in the living state. About 50 X.

of foamy virus (Enders, and Peebles, 1954; Rustigan *et al.*, 1955) obtained from our uninoculated monkey kidney cultures. When Enders medium was used in Hep-2 cultures infected with foamy virus, large syncytial masses appeared. These eventually in-

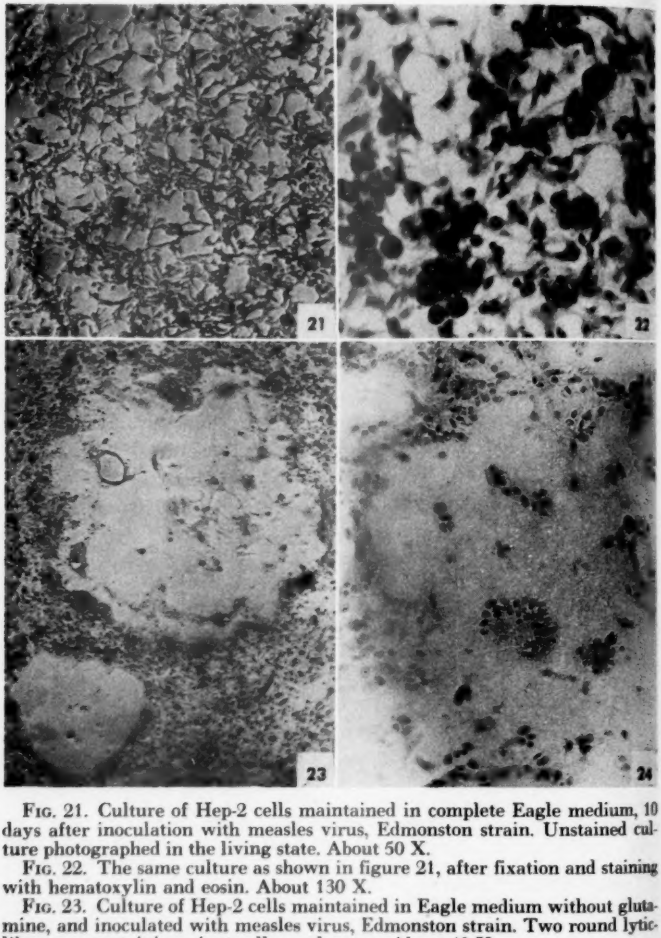


FIG. 21. Culture of Hep-2 cells maintained in complete Eagle medium, 10 days after inoculation with measles virus, Edmonston strain. Unstained culture photographed in the living state. About 50 X.

FIG. 22. The same culture as shown in figure 21, after fixation and staining with hematoxylin and eosin. About 130 X.

FIG. 23. Culture of Hep-2 cells maintained in Eagle medium without glutamine, and inoculated with measles virus, Edmonston strain. Two round lytic-like areas containing giant cells can be seen. About 40 X.

FIG. 24. Culture similar to that of figure 23, after fixation and staining with hemotoxylin and eosin, showing clusters of nuclei in a giant cell in a lytic-like area. About 120 X.

volved the whole culture, and finally caused its complete degeneration. In cultures maintained in Eagle medium, containing glutamine, only small foci of degeneration were seen, consisting of small clumps of rounded cells and very few syncytial masses. With this last medium, complete destruction of the inoculated cell sheet was not found in a 60-day observation period.

TABLE 9

Effect of Glutamine on Morphological Pattern of Cell Degeneration Induced by Measles Virus

Individual Cell Degeneration	Multinucleated Cells
Complete Eagle medium	Eagle medium *minus* glutamine
Enders medium *plus* glutamine	Enders medium (amniotic fluid, embryo extract, serum)

Closing Remarks

It is evident that no general theory can yet be advanced to explain the propagation of animal viruses. Many of the factors which influence virus proliferation and cell response are often stumbled upon, rather than arrived at by inference. For example, the formation of multinucleated giant cells in measles virus infected cultures deprived of glutamine was discovered by accident, when a member of the laboratory made up a batch of Eagle medium and forgot to include the glutamine component. However, certain fundamental approaches are required in the elucidation of this problem, and the recent introductions of a quantitative assay by plaque counting and of host cells grown under well-defined conditions, are permitting these developments to go forward at an ever-increasing pace. Some of these developments in which our laboratory in New Haven is engaged have been reviewed in this paper as examples of current approaches. These included: (1) adsorption and growth of viruses in cells of differing levels of susceptibility, (2) maturation and release of virus from cells grown in serum-containing and in synthetic media, and (3) effect of cell nutrients on cytopathic responses. The studies on poliovirus proliferation under strictly defined conditions illustrate that virus synthesis does not occur automatically once the virus reaches the cell, but may be controlled, either

favorably or unfavorably, by compounds normally found within cells. The ratio and concentration of these compounds may well be the deciding factor which determines the rate at which virus synthesis proceeds.

Discussion

Dr. DeCarvalho: I would like to ask Dr. Melnick if he has had any experience on the human amnion cultures or similar work, and what is the quality of glass and how much calcium is added to the medium?

Dr. Sabin: Would Dr. Melnick care to express some more philosophic opinion based on these very beautiful data he has presented. In other words, what bearing does this new knowledge of the factors of proliferation of viruses and cells have on the approaches to the study of the relation between virus and cancer?

Dr. Melnick: The work on cell growth in synthetic medium was done with monkey kidney cells. Although we have used amnion cells in the laboratory, they were not used for the studies indicated. The amounts of calcium required in the protein-free medium were about three or four times that present in the conventional salt solutions. As for the glass, Dr. Rappaport has found that the soft glass used in the Brockway prescription bottle will allow monkey kidney cells to grow readily in synthetic medium, but that certain grades of the pyrex bottles will not allow the cells to grow. Some grades of pyrex glass may be prepared for growing cells in SM-2 by washing with alkali, but other grades were unable to be treated in any way so that the cells could grow on their surface. It appears that a cell coated with protein will grow on any glass coated with protein; but if the protein is not present, then the catonic bonds that are present between cells and glass become important factors.

Dr. Goodpasture: Does the difference in glutamine have any effect on the intranuclear inclusions of the measles?

Dr. Melnick: Inclusions could not be found regularly in Hep-2 cells infected with measles, regardless of the presence or absence of glutamine. Inclusions depend somewhat on the type of cell that is involved. In monkey kidney cells infected with measles virus, for example, it is rare to find a cell which does not have

intranuclear inclusions. In the human epidermoid cell it is not uncommon to find many cells without any inclusions even though they have the characteristic multinuclear appearance.

Dr. Sabin's question is one that might be discussed with more profit at the end of this Symposium.

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FACTORS INFLUENCING PROLIFERATION OF VIRUSES IN NORMAL AND MALIGNANT CELLS

(ABSTRACT)

GEORGE O. GEY*

We have learned to dissect the organism of its component living cells at any stage of its development from conception through all stages of life. Many of such isolated cells may be propagated as cultures in a variety of ways, even diseased tissues lend themselves almost equally as well to such dissections. The isolation and control of various types of living cells in tissue culture would appear to provide ideal subjects for the study of cellular responses to viruses in relation to cancer. In our laboratories at The Johns Hopkins Medical Institutions, Dr. Bang and the author, with other collaborators, have been concerned with a direct evaluation, not only of host-cell responses to tumor viruses in continuous cultures, but also of the role which viruses and other factors may play in controlling normal growth or altering the growth of cells in various directions. In this presentation, we will stress the importance of an understanding of the stability of various cell strains of animal and human origin which are so widely used throughout the world today. These have assumed increasing importance as tools in basic science and in public health. The possible role which viruses and other extrinsic factors play alone or in combination in setting the mutational stages leading to various cellular alterations and even of conversions to cancer cells will be discussed. The many new techniques available and the direction in which others should be developed for a better evaluation of total growth and of abnormal cellular responses, especially those of a qualitative and quantitative nature in relation to cancer will be emphasized. In the category of what we see of viruses in cells, we need a better evaluation of the morphological display in relation to biological activity.

* Director, Finney-Howell Cancer Research Laboratory, Department of Surgery, The Johns Hopkins Medical Institutions, Baltimore, Maryland.

Discussion

Dr. Sabin: Dr. Gey had a very difficult assignment. His title was "Factors Influencing Proliferation of Tumor-Inducing Viruses," and as he concluded his paper he said that he did not have many to work with. And so, instead, he gave us a great deal of his rich experience on the effect of certain other viruses, particularly Eastern Equine on various tumor lines. But, it seems to me, that the problem being faced here is not so much dependent upon observations of what cytopathogenic viruses do to cells in tissue culture, but rather what viruses which may merely stimulate cells to grow do to cells without destroying them. Now I would like to ask Dr. Gey whether he thinks that, on the basis of present knowledge and particularly in the light of his own accumulated experience over the years, is it possible to differentiate a malignant cell from a normal cell in tissue culture to the point where tissue cultures might be used for experimental induction of malignant changes? Just how readily can we, at the present time, determine whether or not a malignant change has been produced in tissue culture, beyond that which has occurred spontaneously apparently on a number of occasions that Dr. Gey knows about?

Dr. DeCarvalho, Cleveland: In connection with that same question, I would like to ask Dr. Gey what he can tell us of the A.V. strain of human amnion. Sometimes, one in a hundred, we find a culture of human amnion growth in the same conditions, media and glass and all, that the pattern of growth is entirely different. Instead of a sheath of cells we found clones or tumor-like formations of the amnion cells. How could this be explained in the experience of Dr. Gey?

Dr. Syverton: Dr. Gey, over many years has cautioned investigators employing animal cells in culture of the potential dangers of unwittingly picking up a contaminating virus from ingredients in the cell culture media. Dr. Gey showed today a cell injured in manner entirely similar to the injury that results from infection by either simian foamy virus or the Edmonston measles strain. Since it is not difficult to establish proof for contamination of a culture by demonstration of extraneous virus upon transfer to wide variety of cells now available, I should like to ask Dr. Gey whether he obtained evidence by use of cell cultures or by trans-

fer to animals that the cytopathogenic changes attributed today as possibly resulting from infection came from extraneous virus infection.

Dr. Gey: To answer your questions in the best way that I can, I think I would say this: Whatever techniques we used to better delineate normal from malignant cells, certainly the cells that we have had that were stable, show at least a dozen differences between their malignant derivative type, and the normal prototype. If a change were to occur in the normal cells derived from one and the same host, no matter what that change would be, I would say this is just one of the things that a normal cell can do. It can become something else other than a tumor cell; it can mutate in many directions; it can differentiate just as you differentiated from a single cell. I think I've seen good evidence of this differentiation in many tissue cultures, where a cell of one type becomes transformed into another. We must accept the fact that cells can change from one type to another. But we cannot easily declare them as malignant or not malignant. The only challenge that anyone is willing to accept is the one that was defined this morning. I would agree that a metastasizing tumor, or a tumor invading normal tissue, is all the evidence that we need. But how are we going to get this in the human? That is, of course, up to persons like Dr. Moore. But I would say in connection with animals that we have all the evidence that we need. The cell lines that were altered in cultures were put in animals; they produced tumors, and they killed the animals. Now, other altered lines did not do this at all. Not only that, but we have another situation in culture for which we must account. We have lines which are so different from their normal prototype that even after years of transplantation, they were shown to be consistently the same type of cell. Then there appeared on the scene —an entirely new event, the cells which were so greatly altered were no longer transplantable. Now we have a right to assume that the host in which we were inoculating these cells would mutate. We have the right to assume that the tumor cells could further mutate into new cells which lack, let us say, necessary properties of transplantability. Or we might say that this tumor cell had picked up *in vitro* a virus, which, when this cell was put into a new host, promptly destroyed the tumor cell.

Dr. Syverton: Yes. You pointed out cells that had undergone cytopathologic alteration not unlike the cytopathogenic effect from infection by foamy or measles viruses. Two questions: a) did you demonstrate either of these foamy virus or measles virus by transfer to other cultures, monkey or human?; b) have you ever recovered an extraneous carrier virus from cells kept by continuous culture? In common with many other laboratories, we have not encountered an extraneous virus in cells kept under continuous culture, despite use over many years of ingredients in our cell culture media that were potential sources of such extraneous viruses, nor have we encountered by use of adult pooled sera evidence for transmissible cytopathogenic effects. We, of course, find an occasional sample of serum that gives evidence for toxicity. It is surprising that blood-borne orphan viruses are not encountered.

Dr. Sabin: Can it be said that there is unequivocal evidence that of the various changes you have demonstrated in tissue cultures, are due, in fact, to spontaneous viruses, no matter what their origin may be, whether it be the medium, or whatever else? Such is the question that Dr. Syverton suggested. For example, in a tissue culture in which you have this foamy type of degeneration have you been able to demonstrate that this foamy type of degeneration transferred to a HeLa cell is a transmissible agent, as has been shown with the foamy degeneration occurring in monkey kidney? You suspect it—but can you say that there is unequivocal evidence that any of those changes are due to spontaneous viruses, either in the medium or in the cell?

Dr. Gey: There are any number of ways that one can move in this tissue culture business. I have not seen evidence that a tumor virus has increased the proliferative response of cells in culture. I am familar only with the work of Dr. Wilson Lo, who has worked with Dr. Bang and me, and of other works of Smith and Kun concerned with tumor viruses such as Rous and myxoma virus and of the effects of such viruses as herpes, vaccinia, New Castle, and swine flu on the cells of the chorio-allantoic membrane. In this latter work, evidence was obtained on an increase in anaerobic hexosediphosphate dismutation with no specific metabolic activity delineating the tumor viruses from the others. In the work of Dr. Lo, a production of acid, probably lactic acid,

accompanied the virus infection of the chick cells. If anything, the Rous virus gave good evidence of its cytopathogenic activity with no evidence of a proliferation response and with the production first of giant cells which later disintegrated.

Concerning Dr. DeCarvallo's questions, I am afraid that I have little to offer since I have had only a few experiences with amnion cells. It is my guess that the tumor-like formations of the amnion cells were due to interaction of several cell types producing a tiny rounded mass or organoid. These may be found in many cultures derived from other tissues such as kidney and lung and where there is a continual opportunity for an interaction between the stromal cells and the parenchymatous components. Such mixed cell cultures survive on glass indefinitely.

The so-called "foamy" viruses (including measles), of which Dr. Syverton speaks, may have been encountered many times in our laboratory. We have not carried out extensive efforts to transfer what we have seen to the monkey or to the human. We have, however, seen and passed cultures showing this lesion serially in cultures and even through the ascites form when a tumor strain was passed intraperitoneally in the mouse. It is surprising to me that Dr. Syverton has not obtained real evidence of extraneous virus infection in continuous cultures. I am still inclined to believe, as Dr. Sabin suggests, that what we see is due to spontaneous viruses from original cells or medium.

The evidence that I go by is that of the cell being abnormally stressed. Whether the cell goes through a radiation experience, whether the cell is hit with a carcinogen, whether the cell is hit by a virus—it is my feeling that there is common response to non-specific stress, and this is all that one needs. We should try to isolate single cells, and start with them. We should measure the potentialities of these single cells in terms of their original complement of nuclei or anything else. Now these altered tumor producing cells certainly have a complement of something that does not belong there. If they can recover, they may recover and be in another form. It will require much work to isolate single cells from tissues which are diseased. Where else would one look for evidences of conversions of cells, except in those areas where the tissue is damaged? I am only pointing out that if one examines the earliest specimens he will not see in all

cases where one finds early cancer a perfectly health situation. We see this in many, many cases. It's up to us, as experimental cytologists, to dissect this material in a dynamic way and find out what the potentials are I would like to describe how we intend to proceed with this. Dr. Reimer of our laboratory has devised a means which will allow us to follow the outcome of the proliferitive capacity of any number of cells, from one up to about one thousand cells per cubic millimeter. The technique is exceedingly simple and involves the use of a double cover slip with a space of about two-tenths millimeters between, in which we follow, by film methods, the total history of what a cell does: the ensuing growth rate, the death rate, the identification, if one can, of degrees of ploidy, the inter-mitotic interval, the character of the mitosis, and the other data which we think is significant for morphological display. In this way starting with either polyploid cells from various tissues which we know exist there, or with cells which have another designated composition, we can say where they are going in response to a specific environment. The important thing is the fact that all of these things are under control. This perhaps is the most significant thing in the whole of tumor pathology.

TRANSMISSION OF TUMOR INDUCING AVIAN VIRUSES UNDER NATURAL CONDITIONS

BEN R. BURMESTER*

Neoplastic diseases among animals have taken their greatest toll in the domestic chicken. It has been estimated that one-third to one-half of all mortality among pullets and hens is due to neoplasms (Hutt, 1938; Hays, 1944; Davis and Doyle, 1947; Darcel *et al.*, 1952). The number of deaths from all causes varies considerably from one flock to another and may reach 50 or more per cent during the period of the first laying year.

All kinds of tumors are found in chickens; however, the occurrence of the different types is at marked variance with that found in man and other animals. Detailed studies of 384 neoplasms found in 1,781 chickens sent in for autopsy from 3 different flocks have been reported by Olson and Bullis (1942). The investigators found that the leukotic diseases accounted for 66 per cent of the neoplasms and the remaining 34 per cent was made up of benign and malignant tumors of 20 different cell types. The greater part of the 34 per cent was comprised of leiomyomas, embryonal nephromas, epithelioblastomas, and fibrosarcomas. By far the greater part of the leukoses were lymphoid. Thus they found that 56 per cent of all neoplasms were classified as lymphoid leukosis. Erythroid and myeloid leukosis made up only 5 and 6 per cent, respectively.

Two years earlier Goss (1940) reported on autopsies of 7,408 deaths and found neoplasms in 19.5 per cent. The neoplasms were distributed among 13 different cell types and the leukoses comprised 90 per cent of them. An even higher proportion was reported by Hutt *et al.*, (1941), who stated, "Approximately 95 per cent of deaths attributed to neoplasms were caused by lymphomatosis of one kind or another." These reports serve to illustrate the distribution and relative importance of the different types of neoplasms causing the death of chickens under natural conditions.

* United States Department of Agriculture, Agricultural Research Service. Regional Poultry Research Laboratory, East Lansing, Michigan.

The exceedingly high rate of occurrence of lymphoid leukosis, more generally known as lymphomatosis, is found in all sections of the United States, and with few exceptions in all parts of the world.

The term "lymphomatosis" has been used as the common name for three pathologic forms, namely: ocular, neural, and visceral, the difference being primarily in the organ location of the immature lymphoid elements. In spite of the similarity in cell type, there are many differences in the epizootiology and in other characteristics which indicate that the various forms are caused by different agents (Fenstermacher, 1936; Campbell, 1946, 1954, 1956; Davis and Doyle, 1947; Burmester, 1947, 1952; Cottral *et al.*, 1954; Burmester *et al.*, 1955, 1956).

History

On the basis of the descriptions of disease conditions reported, it would appear that the disease we know today as visceral lymphomatosis (lymphoid leukosis) was found in several parts of the United States during the first decade of this century (Kaupp, 1921; Davis and Doyle, 1947; and Winton *et al.*, 1955). Its occurrence gradually increased during the next three decades, but at a much faster rate than the poultry population, so that it has become the most important disease problem among adult chickens.

The history of neurolymphomatosis is somewhat different. During the second decade, it was found in many eastern states; it then appeared to spread to the midwest and western states so that by the latter part of the third decade, it was responsible for heavy death losses among growing chickens 3 to 8 months of age. Its natural occurrence has since decreased markedly. Data show that whereas the incidence of neurolymphomatosis decreased markedly during the fourth decade, visceral lymphomatosis increased during the same period to a point where it became responsible for two-thirds to three-fourths of all mortality from lymphomatosis (Hoffman and Stover, 1942; Davis and Doyle, 1947). The occurrence of ocular lymphomatosis has generally followed that of neurolymphomatosis.

Similar observations on the spread of these diseases have been made in England (McGaughey and Downie, 1930; Johnston and

Wilson, 1937), in Australia (McLennan, 1935), and in Japan (Emoto and Miyamoto, 1930).

The infectious neoplasms of the chicken exhibit a striking paradox. In spite of the apparent epizootic nature of the lymphoid diseases they have not been easily transmitted in the laboratory and Koch's postulates demonstrated; whereas, erythro- and myeloblastosis and the myxo- and fibrosarcomas, even though their natural rate of occurrence is very low in comparison with lymphomatosis, have been easily propagated in the laboratory. Thus, ocular lymphomatosis has not been reproduced experimentally, and neurolymphomatosis has been transmitted only with cell-containing inoculums, and then only at low rates (Pappenheimer *et al.*, 1929; Furth and Breedis, 1935; Hepding, 1936; Durant and McDougle, 1945). Repeated attempts with filtrates have thus far failed (Furth and Breedis, 1935; Olson, 1937; and Campbell, 1954).

Visceral lymphomatosis was long considered to be a nontransmissible neoplasm (Anderson and Bang, 1928; Mathews and Walkey, 1929; Feldman, 1932; Feldman and Olson, 1934, 1943; Fenstermacher, 1936). Furth and Breedis (1935) were the first to report on a filtrable agent which caused visceral lymphomatosis and occasionally other tumors. Overwhelming evidence for the viral nature of the causative agent of visceral lymphomatosis was obtained at the U. S. Regional Poultry Research Laboratory where inbred lines of chickens, selected for susceptibility to lymphomatosis and freedom from naturally acquired infection, were used (Waters and Prickett, 1944; Waters, 1951). Transmission with tumor homogenates was obtained with about three-fourths of all the naturally occurring tumors tested and about one-half of the tumors were shown to contain a filtrable transmitting agent (Burmester and Prickett, 1945; Burmester and Denington, 1947; Burmester, 1947). After several serial passages, tumor filtrates injected intraperitoneally in amounts of log —8.0 gm. of tumor equivalent and plasma of tumor-bearing birds in volumes of log —4.4 ml., have caused visceral lymphomatosis in 60 per cent or more of the chickens inoculated (Burmester and Gentry, 1956). A similar viral agent causing visceral lymphomatosis was also found in the livers of normal embryos from "carrier" hens, and in the feces and oral washings of hens and their day-old progeny (Cottral *et al.*, 1954; Burmester and

Gentry, 1954a; Burmester *et al.*, 1955; Burmester and Waters, 1956; and Burmester, 1956).

The ease of transmission of erythroblastosis and myeloblastosis by inoculation with cell-free filtrates is attested by the large number of papers reporting studies on its transmissibility and other characteristics of the viral agent of over 30 different strains. (See literature cited by Olsen, 1940; Engelbreth-Holm, 1942; Furth, 1946; Oberling and Guerin, 1954; Beard, 1956; Beaudreau *et al.*, 1956; and others.) Similarly high transmissibility of the fibrosarcomas, as exemplified by the Rous chicken tumor, is well known (Rous, 1911; Foulds, 1934; Bryan, 1955, 1956). Many similar connective tissue tumors have been described, and it would appear that most such tumors of spontaneous origin contain a filtrable transmissible agent (Andrewes, 1933; Duran-Reynals, 1946, and literature cited). In contrast to the ease of experimental transmission by any one of several parenteral routes, no one has experimentally transmitted any connective tissue tumor or erythro- or myeloblastosis by methods other than parenteral inoculation. Evidence of direct or indirect contact transmission, or of transmission via the egg has thus far been uniformly negative (Anderson and Bang, 1928; Jarmi, 1930; Claude and Murphy, 1933; Carr, 1944; and Eckert *et al.*, 1951). Evidence of a contagion causing these neoplasms under natural conditions is also lacking with the exception that under certain conditions erythroblastosis has occurred in epidemic proportions (Hamilton and Sawyer, 1939; Wickware, 1946; and Hadlow, 1955). No explanation has been offered for such isolated instances of its occurrence in almost all chickens of some pens while chickens of other pens of the same flock are entirely free of it.

It is readily apparent that some of the avian neoplastic viruses are contagious and others of similar or greater infectivity are not. There is also evidence indicating that certain strains may vary greatly between several passages in this characteristic (Burmester and Gentry, 1954b). It is obvious that the ability to cause disease, under natural conditions, is a characteristic separable from infectivity. This ability enables the agent to cause epizootics and, in general, maintain itself in nature. It is, therefore, the most important single characteristic of a disease agent and,

yet, almost nothing is known concerning its nature and the factors influencing it.

Natural Transmission of Lymphomatosis

Observations on the natural occurrence and spread of lymphomatosis suggest that natural infection may occur (1) through the egg from infected parents to the offspring; (2) by contact with air, feed, water, equipment, or caretakers that have become contaminated with infected secretions, excretions, or droplet nuclei; or (3) by direct contact of infected with non-infected birds.

Egg transmission.—It was early suggested that neural lymphomatosis was transmitted by the egg (Doyle, 1928). Indirect evidence in support of this mode of transmission has since been reported by many investigators. Some introduced hatching eggs only into isolated areas where chickens had not previously been reared, and obtained a high incidence of the disease (McLennan, 1935; Emoto and Miyamoto, 1930; Marginson and McGaughey, 1931; Warrack, and Dalling, 1932; Hepding, 1936; and Waters, 1945). Results of incubator exposure experiments and other transmission studies were also in support of egg transmission (McClary and Upp, 1939; Durant and McDougle, 1945; Waters and Bywaters, 1949). In contrast to this view, the importance of this mode of transmission was minimized and, later, evidence was presented indicating that neurolymphomatosis was not transmitted through the egg (Cole, 1949; Cole and Hutt, 1951).

Direct evidence that the agent of visceral lymphomatosis (V.L.) is present in the egg was only recently obtained (Cottral *et al.*, 1954). Infectivity tests of embryo liver suspensions demonstrated the presence of the V.L. virus in eggs of certain dams and its absence in eggs of others. In subsequent experiments (Burmester *et al.*, 1955), 22 hens were selected at random from an infected flock of White Leghorn chickens. Of these, 17 were from two susceptible inbred lines, and five were from a line selected for resistance to lymphomatosis. Birds of all lines were reared together, thus being exposed to the same environment during the same period of time. The presence of the virus in 15-day embryos from embryonating eggs of each dam was determined by inoculating groups of 40 to 50-day-old chicks of a susceptible line with the embryo liver preparations and observ-

ing the occurrence of lymphomatosis. It was found that 16 of the 17 hens from the two susceptible inbred lines laid eggs which, when incubated to 15 days, contained enough virus to produce a significant incidence of V.L. in inoculated chickens. In contrast, only one of five hens from the resistant line showed evidence of shedding the virus into their eggs. These tests were made while the hens were about one year of age. Similar tests were conducted on eggs laid 3 months later, and on others laid 6 months later. Evidence of some variation in shedding of the virus by individual hens was obtained, but a generally high level of virus in the eggs was evident for hens of the 2 susceptible lines. However, tests made on eggs laid when the same hens were 2 years and 3 years of age, gave transmission results indicative of a much lower level of virus.

Progeny of the hens of the susceptible lines were hatched from eggs laid when the dams were about 1 year of age. Infectivity tests conducted on the eggs laid by the progeny when they were 8 to 9 months of age, showed that nine of 14 progeny shed virus into their eggs. Subsequent tests of eggs laid when the progeny were about 1 year and about 2 years of age, gave much lower rates of transmission.

It should be emphasized that all hens were apparently in normal health at the time the eggs for infectivity tests were laid. Furthermore, during the year following the laying of eggs for the first test of the original 22 hens, eight hens died of various causes. The death of only three of these was attributed to lymphomatosis; one of them died within 2 weeks and the other two almost a year after the eggs for the infectivity tests were laid. Eggs infected naturally or by inoculation hatch well, and the chicks have high viability (Gentry and Burmester, 1955).

These results demonstrate quite conclusively that the virus of V.L. is present in eggs, while the hens, the embryos, and the chicks appear normal and in good health. Since it has been shown (Gentry and Burmester, 1955) that virus in embryonating eggs propagates during incubation, the response obtained in chicks inoculated with 15-day embryonic liver was the result of virus deposited by the hen and then propagated during 15 days of embryonic development. However, it would appear that the original amount deposited by the hen is of importance in influencing the amount of virus detectable at the fifteenth day. Data

show that genetic constitution and age appear to be important factors influencing the deposition of virus in eggs of hens of an infected flock (Burmester *et al.*, 1955; Burmester and Waters, 1956). Young hens of a line selected for susceptibility to lymphomatosis were more apt to shed significant amounts of virus into their eggs than old hens or those of resistant lines.

Although both neural and visceral lymphomatosis occurred in the flock with the hens that supplied the eggs for infectivity test, only V.L. occurred to any significant extent in the test chicks inoculated, thus adding further to a large accumulation of data (Burmester, 1947; Davis and Doyle, 1947; Waters, 1954; Burmester and Gentry, 1954a, b; Burmester *et al.*, 1956) indicating that the causative agent of neural lymphomatosis is different from that causing visceral lymphomatosis. The lack of evidence for the presence of the causative agent of neural lymphomatosis in eggs of hens from the infected flock is in agreement with the results obtained by workers at Cornell University (Hutt and Cole, 1947a, b; Cole, 1949; Cole and Hutt, 1951). They reported no evidence for egg transmission of "leucosis." A breakdown of their diagnoses at necropsy shows that over three-fourths of all cases of lymphomatosis were of the neural form (Hutt and Cole, 1953). Hence, interpretations and conclusions based on such data should be confined to neural lymphomatosis (Waters, 1954). Contrary to the first suggestion of egg transmission and early confirmatory reports, which were based largely on neural lymphomatosis, the more recent data (Hutt and Cole, 1947a, b; Cole, 1949; Cole and Hutt, 1951; Hutt and Cole, 1953; Cottral *et al.*, 1954; Burmester *et al.*, 1955; Burmester and Waters, 1956) are negative with respect to egg transmission of the neural form of the disease.

Although it has now been shown beyond question that hens shed the V.L. virus in eggs, and that their embryos become infected and provide a medium for the multiplication of the virus, one must assess the significance of this in relation to the natural transmission of the disease.

The occurrence of V.L. in 2 groups of progenies of hens that had been classified with respect to shedding of virus into their eggs, was studied (Burmester *et al.*, 1955; Burmester and Waters, 1955). Both groups were obtained from dams at an age when twelve were classified as heavy shedders, five as light shedders,

and five others as nonshedders. One group of progenies of about ten birds per dam were hatched and reared together in regular incubators and brooder rooms, allowing contact between families, with caretakers and the usual equipment of the incubator and brooder unit. The second group of about 15 birds per dam were incubated and hatched in special family isolation units and then brooded to 3 months of age in isolation cubicles, after which they were moved to regular pens, and held to 500 days of age.

The incidence of V.L. in the first set of progenies was generally higher than that in progenies that were reared in isolation. However, both sets gave similar results in that there was no apparent relationship between the amount of virus in the embryos of specific dams and the occurrence of V.L. in the progenies of the same dams. Thus it was found (Burmester *et al.*, 1955; Burmester and Waters, 1955) that many hens which were classified as heavy shedders produced progenies, none of which developed lymphomatosis during a 500-day period. Furthermore, some progeny from dams that had been classified as non-shedders developed lymphomatosis. Therefore, the presence or absence of the V.L. virus in embryos is not the most important factor in determining whether such chickens, when hatched, will later develop lymphomatosis.

It has been suggested that the unexpectedly low incidence of V.L. in the progeny of shedder dams was, in part, due to the passage of maternal antibodies to the chick via the egg, as occurs in several other chicken diseases. An adequate basis for this suggestion has been provided by the demonstration that when hens were given repeated injections of the V.L. virus they developed neutralizing antibodies (Burmester, 1955a). The progeny obtained after the injection were much more resistant to challenge inoculation than were the progeny of the same hens obtained before the immunizing injection (Burmester, 1955b; Burmester *et al.*, 1956). Neutralizing antibodies have also been found to be present in the serum of day-old chicks hatched of eggs from hens that had received immunizing injections and from hens that had been naturally infected and shown previously to have shed the V.L. virus into their eggs (Burmester, 1957).

Contact transmission.—The importance of the egg transmitted virus becomes clear when we consider results of experiments on contact transmission in which baby chicks of an infected flock

were used as the sole source of infection in exposing chicks from a non-infected source.

Numerous investigators (Warrack and Dalling, 1932; Patterson, 1936; Johnson and Wilson, 1937; Barber, 1942; Waters, 1945; Waters and Bywaters, 1949; Burmester and Gentry, 1954b; Burmester and Waters, 1955) have shown that chicks of a relatively disease-free source, when reared in the same brooder units with other chicks from an infected flock, develop a high incidence of lymphomatosis which is usually greater than the incidence in the progeny of the infected flock. Age at exposure appears to be important since it has been found (Lee *et al.*, 1937, 1944; Hamilton and Bearse, 1946; Waters and Bywaters, 1949; Burmester and Gentry, 1954b) that in any one experiment, the younger the birds at time of exposure, the greater the incidence of disease. Furthermore, the virus of V.L. was found to be present in debris of an incubator that had been used to hatch chicks of an infected flock. When chicks of susceptible disease-free parents were hatched in the same incubator with chicks from infected parents, a high incidence of lymphomatosis occurred in the chicks of the disease-free hen (Waters and Bywaters, 1949; Burmester and Waters, 1955).

Transmission from inoculated birds to non-inoculated penmates has been repeatedly reported (Patterson *et al.*, 1932; Lee *et al.*, 1937; Brandly *et al.*, 1942; Lee *et al.*, 1944; Burmester and Gentry, 1954b; Brewer and Brownstein, 1946; Rao *et al.*, 1950). Factors that appear to be important in contributing to a high rate of contact transmission are: intimate contact of chickens of similar age, young age at exposure, and a high proportion of infected chicks serving as the source of exposure.

The mechanism of contact transmission has also received some attention. It was found that chickens may be infected through any of the natural body openings and, as a result, a portion of the infected birds may die of lymphomatosis. Different routes of infection with the strain RPL 12 V.L. virus, as determined by experimentation, gave levels of transmission in the following descending order: tracheal, nasal, cloacal, conjunctival, oral, and aerogenic. However, when the virus was deposited in the crop, V.L. did not occur at a significant level (Burmester and Gentry, 1954c).

Possible natural sources of infection have also been investi-

gated. Oral washings from naturally occurring and experimentally induced cases of V.L. have been found to be highly infectious. Oral washings of some normal appearing birds of an infected flock, and the oral washings of day-old chicks that were the progeny of hens of an infected flock, also were infectious, although somewhat less so than the washings from lymphomatous chickens (Burmester and Gentry, 1954a Burmester, 1956).

Information on the relation between age and the shedding of V.L. virus in the saliva was obtained in an experiment in which one group of day-old chicks of a susceptible line, free from prior infection, was inoculated intraperitoneally with a strain of V.L. virus. A second group of the same source and age was placed in the same brooder unit. From each group, twelve chickens were randomly selected and oral washings collected at 10, 30, 90, and 180 days of age. The individual washings for each group of chickens and for each collection were pooled and infectivity tests were conducted. The oral washings from the inoculated chickens collected at 10 days were found to be highly infectious and remained so for all collection periods; whereas, tests on oral washings of the contact chickens showed that the 90-day collection was the first to contain significant levels of virus, and the 180-day collection had about the same level as those of the inoculated chickens. Of the twelve inoculated birds, seven died of V.L. during the age period of 98–236 days, and only four of twelve contact birds died during the 206–212 days age period (Burmester, 1956). These results definitely show that chickens may shed highly infectious levels of V.L. virus into their saliva soon after they have become exposed. When the exposure is definite and massive, as by inoculation, large amounts of virus appear in the saliva within ten days. When the exposure is indefinite as by contact infection, a longer period is required for the average level of salivary virus to attain highly infectious levels.

It is significant that the saliva becomes infectious early in the life of the chicken, *i.e.*, in baby chicks when infection is derived from the egg and within 3 months when the infection is derived during the brooding period; and that the chicken remains in apparent good health for long periods thereafter. The feces are also infectious under similar conditions, but the apparent amount of

virus is not as great as in the saliva, and it is less regular in its presence.

Preliminary tests have shown that hens that shed V.L. virus into their eggs and saliva, and day-old chicks hatched of eggs from such hens and which likewise shed virus into their saliva, also may have significant levels of serum antibodies (Burmester, 1957) suggesting little or no interference between serum antibody and the shedding of virus into the egg or in the saliva.

The data indicate that the virus of V.L. is spread by normal appearing hens via the feces or droppings, by the saliva, and in the egg; likewise, chickens with lymphoid tumors shed virus in the same manner. Baby chicks hatching from infected eggs cause a contamination of the incubator by a dissemination of dried extraembryonic fluids, feces, and other hatching egg debris. Such chicks shed virus in their saliva and feces during the brooding period and contaminate the feed, water, and other material and equipment of the brooder units; thus providing an intimate source of infection for susceptible penmates.

Since overt infection occurs after the experimental administration of virus by the oral and respiratory routes, it is likely that these routes are portals of entry of naturally disseminated virus.

Evidence that the common chick drinking fountain of the brooder unit is of major importance in the spread of the disease is shown by the results of an experiment wherein the influence of two different types of water fountains on contact transmission was studied (Burmester, 1957). It was found that the extent of transmission between chicks inoculated intraperitoneally and noninoculated chicks of the same hatch, when brooded in a pen with a rapidly flowing water fountain, was only about one-third as great as the transmission between birds reared in a similar pen but with a non-flowing water fountain. Virus placed directly in a non-flowing water fountain (5 times during the first 30 hours) of a third pen produced as much V.L. as occurred in the contact chicks of the non-flowing water pen. These results strongly indicate that chicks, infected at one day of age by intraperitoneal inoculation, shed sufficient virus in their saliva to cause a significant V.L. virus contamination of drinking fountains when the fountain is of the reservoir type and contamination is allowed to accumulate. It seems likely that the water in

such fountains is responsible for the spread of a significant amount of V.L. virus to susceptible, previously non-infected chickens of the same pen. It is probable that under natural conditions this mechanism of the spread of infection is of importance since it has been shown that day-old chicks of naturally infected hens shed virus in their saliva.

Natural Transmission of Other Neoplasms

Almost nothing is known concerning the natural transmission of the viruses causing the noncontagious though infectious neoplasms of the chicken; however, related information has led to certain speculations. Recent data (Beard, 1957) indicate that the viruses of erythroblastosis, myeloblastosis, visceral lymphomatosis, and Rous sarcoma are serologically related. Furthermore, many investigators (Burmester 1952, and literature cited) have obtained, during the serial passage of a neoplasm of a certain type, an occasional occurrence of neoplasms of other types. Thus, erythroblastosis, endotheliomas, and various sarcomas, have occurred in inoculations with material from cases of lymphomatosis; the occurrence of endotheliomas and various kinds of sarcomas in inoculations with erythro- and myeloblastosis has often been reported. There is some suggestion of a sequential tendency in the occurrence of these variations. On the basis of these observations, together with those on the natural transmission of the various avian neoplasms, it may be suggested that the natural occurrence of the infectious neoplasms of the chicken, which are not contagious, may be explained by changes such as mutations of the visceral lymphomatosis virus. These mutations result in agents which have lost their ability to spread to a new host and which produce different types of neoplasms. Thus, such neoplasms are a "dead end" in natural transmission and analogous to "blind alleys" which may account for their very low natural occurrence in comparison with visceral lymphomatosis. The latter must then be considered the "wild type," the source of all other known avian neoplastic viruses, and responsible for their continued natural occurrence at a very low rate. All are likely to be related serologically to a varying degree, but have definite distinctive features which require them to be classified as different entities.

Summary

Neoplasms account for a high proportion of the mortality among domestic chickens. This is primarily due to the incidence of visceral lymphomatosis (lymphoid leukosis) which appears to be the only one of several infectious neoplasms of the chicken which is also contagious. This disease was first reported to occur occasionally in several parts of the United States during the first decade of this century. Its occurrence gradually increased so that it has become the most important disease problem in adult poultry.

Most infections with visceral lymphomatosis remain in a latent state. Many chickens or only a few in a flock may die of the disease, yet most of them shed virus into their eggs, saliva, and feces. Such chickens also have circulating antibodies which are deposited in the egg and serve to give a certain measure of protection to their progeny. These chicks shed virus in their saliva and feces, contaminating the hatching units of the incubator and the brooder units, thus providing a source of infection for other, more susceptible chicks in the same units, and the progeny of hens without an appreciable antibody level. Experiments indicate that the drinking water may play an important role in the mechanism of transmission within the brooder units.

The occurrence of other infectious, though non-contagious, avian neoplasms under natural conditions may be related to the close interrelationship between their causative viruses and the virus causing visceral lymphomatosis.

Discussion

Dr. Sabin: These interesting findings present a beautiful model for a virus causing acute infectious disease, despite the fact that it has a somewhat long incubation period and that "tumors" are a regular manifestation of the process. Its behavior is entirely similar to the classical viruses which cause acute infections. One may wonder, therefore, to what extent the knowledge obtained with this virus can serve as a model for potential tumor viruses —malignant tumor viruses—which do not behave like infectious diseases. Would anyone here care to challenge the concept that visceral lymphomatosis of chickens has anything to do with ordinary malignant tumors as we know them in mammals?

Dr. Schmeisser, Professor Emeritus, University of Tennessee: It gives me great pleasure to have heard this last paper, because in 1914 I published the second transmission of leukemia in the fowl in the world's history. The first was transmitted by two veterinarians, Ellerman and Bang in Norway. The second transmission, with an entirely new strain, was committed in this country at the Johns Hopkins, under the guidance of Dr. Welch. This confirmed Ellerman and Bang's transmission. Our interest in the present transmission reported is that in the fowl the lymphocyte responds most commonly. The early transmissions were certainly myeloblastic. Those of Bang and those of my strain, which were transmitted through 14 generations, were always myeloblastic. We reproduced in the fowl all the changes then known in human leukemia. The blood picture is very striking. We had mitotic figures in white cells, and red cells in the streaming blood; the characteristic changes in the liver and the spleen were quite similar to those of human beings in type, myeloblastic. It was transmitted first by ordinary elmusions from the liver and spleen into healthy fowl. We inoculated in the vein of the wing and waited. About 6 weeks later those that became sick were anemic and weak. Studying the flowing blood, we saw the different changes and they were indeed a very typical procedure. Streaming blood when introduced into the blood stream of fresh animals reproduced the disease. We also had tumor-like formations occurring about the head and on the surface of the body which were different from the usual disseminated leukemia type of blood infection.

Dr. Dmochowski: In view of some observations which I will mention tomorrow, I would like to ask Dr. Burmester if he could perhaps expand a little and express an opinion on the relationship between the lymphomatosis and Rous sarcoma etiological agents.

Dr. Burmester: In cooperation with Dr. Ray Bryan at the Institute, and also with Dr. Beard at Duke University, we have been doing some serological work that Dr. Beard has already reported on. It would appear that immunologically there is a close relation between the virus of visceral lymphomatosis, the Rous virus, and also the viruses causing erythroblastosis and myeloblastosis. These viruses apparently are distinct entities; they have different

characteristics and they produce distinctly different diseases. It's very consistent. Yet they are immunologically related, which, of course, brings up a possible point as to how these viruses get around in nature. We find that erythroblastosis and myeloblastosis occur naturally at a very low rate, and as I have indicated, no one has demonstrated experimentally that these viruses are transmitted by contact or by insect vectors. We have found that upon passage of the visceral lymphomatosis virus that we at times get an occurrence of other neoplasms such as erythroblastosis and myeloblastosis and also carcomas. It would appear that the visceral lymphomatosis virus is quite labile. Apparently it mutates and causes the formation of these other neoplasms. We have often wondered whether or not this may explain the occurrence of these other neoplasms in nature, since the visceral lymphomatosis virus is the only one of the group that is contagious.

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ENHANCEMENT OF SUSCEPTIBILITY TO VIRUSES IN NEOPLASTIC TISSUES

HILARY KOPROWSKI,* ROBERT LOVE,† AND IRENA KOPROWSKA‡

Every dog has his day. So does the virus, particularly when it encounters a tumor in which it can multiply and eventually destroy it. But not all types of tumors will support virus growth and only certain types of viruses will thrive on malignant cells.

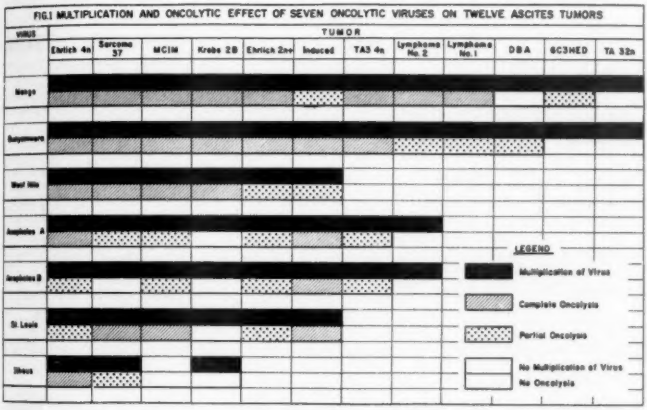


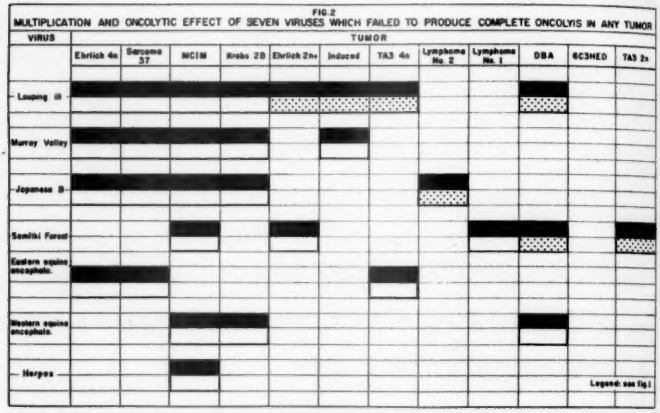
FIG. 1 MULTIPLICATION AND ONCOLYTIC EFFECT OF SEVEN ONCOLYTIC VIRUSES ON TWELVE ASCITES TUMORS

Figure 1 shows the range of activity of seven viruses in twelve ascites tumors of mice. Mengo and Bunyamwera viruses multiplied in all of the tumors tested. Mengo caused complete oncolysis of eight tumors and partial oncolysis of two; Bunyamwera infection had an oncolytic effect on seven and caused partial oncolysis of three tumor lines. West Nile produced oncolysis of four tumors. Anopheles A and Anopheles B viruses multiplied in eight tumors but Anopheles A caused complete oncolysis in only two and Anopheles B only in one tumor. Six tumors sup-

* The Wistar Institute, Philadelphia, Pennsylvania.
† The National Cancer Institute, National Institutes of Health, U.S.P.H.S., Bethesda, Maryland.
‡ Department of Pathology, University of the State of New York, Medical Center of New York City, Brooklyn, New York.

ported growth of the St. Louis virus and three of the Ilheus virus. Oncolysis was observed only in three instances with the St. Louis virus and only in one with the Ilheus virus.

Figure 2 indicates viral agents which multiply in some of the



tumors but fail to cause discernible oncolytic effect. With the Eastern, Western equine encephalomyelitis and Semliki Forest viruses a low level of multiplication was occasionally observed but the results were of equivocal character and oncolytic effect was totally absent.

Apart from the tumors listed in these two figures, rat hepatoma, Yoshida sarcoma and P288 tumor of dba mice were also investigated for their susceptibility and all were found to be completely resistant to any viral infection. On the other hand viruses such as of dengue, yellow fever, pseudorabies, rabies, Ntaya and mouse adapted Type II polio-virus had no affinity for any of the tumors used in these investigations.

Effect of Different Concentrations of Virus

In the preliminary experiments massive doses of virus were used in order to determine their oncolytic activity. It became of interest to verify whether such an effect would still be discernible when smaller concentrations of viral agents were injected into tumor-bearing mice.

In experiments with the West Nile virus PR1 strain of mice was used because of their resistance to the lethal action of the virus (1). As shown in table 1 groups of mice were implanted with 5 million Ehrlich ascites tumor cells and four days later were injected with decreasing five-fold dilutions of the West Nile

TABLE 1

Effect of Different Concentrations of West Nile Virus Upon Ehrlich Ascites Tumor

Virus Inoculum Mouse LD_{50}*	Exp.	Mortality of Mice† Per Cent	Average Day of Death	LD_{50} Titer* of Ascites Fluid	Oncolysis	
					Days After Virus	Biossay Tumor Ratio
4.85	A	57	11 ± 0.95	N.T.	9	N.D.
	B	44	49 ± 15.5	>6.00	13	0/6
4.15	A	33	6 ± 0	4.50	9	0/6
	B	37	41 ± 0	6.00	10	1/4
3.45	A	16	12 ± 0	N.T.	9	N.D.
	B	25	45 ± 3.7	1.50	13	0/6
2.75	A	20	12 ± 0	3.50	7	N.D.
	B	75	35 ± 8	4.00	8	0/6
2.05	A	66	21 ± 11.5	N.T.	8	N.D.
	B	75	30 ± 10.2	>6.50	13	0/6
1.35	B	75	31 ± 10.4	5.75	13	0/6
Controls	A&B	100	14 ± 3	...	..	6/6

* Expressed as Log. to the base of ten.
† PRI strain of mice.

virus in form of infected mouse brain suspension. All of the control animals died from malignancy with an average day of death at 14 ± 3. In the virus-treated animals of Experiments A and B oncolysis was observed in all instances, as indicated by the survival rate of mice and by microscopic and biological evidence of oncolysis. The virus multiplied in the ascites fluid as indicated by the LD_{50} titer in mice. Those of the virus-treated mice which finally succumbed to the malignant process, did so after a prolonged incubation period. The two experiments gave essentially the same results although the average day of death for the virus-treated mice in Exp. A was lower. Even though the antitumor

effect was observed with all the dilutions injected, there appeared to be an optimum oncolytic dose which represented neither the highest nor the lowest concentration of virus. For instance, in both Experiments A and B $10^{3.45}$ mouse LD_{50} of virus protected more mice than any other dilution of virus.

In another experiment along the same lines, Bunyamwera virus was used as oncolytic agent (2, 3). ICR mice were implanted with 32 and 12 million Ehrlich ascites cells, respectively, and 4 days later decreasing 5-fold or 10-fold dilutions of Bunyamwera virus were injected intraperitoneally. Since Bunyamwera virus is lethal to any strain of mice, oncolytic effect was judged by examination of cells on smears obtained from peritoneal cavity and by means of bioassay (Table 2). The results indicate

TABLE 2

Effect of Different Concentrations of Bunyamwera Virus Upon Ehrlich Ascites

Tumor Inoculum No. of Cells x 10^6	Virus Inoculum Mouse LD_{50}*	Virus Titer† of Ascitic Fluid	Oncolysis	
			Days After Virus	Bioassay Tumor Ratio
	4.7	4.50	2	0/0
	4.0	5.50	2	0/4
32	3.3	6.50	2	0/4
	2.6	6.75	3	0/4
	1.9	6.50	1, 2, 3	0/0
	None	...	.	12/12
	2.25	6.50	4	0/8
	1.25	6.50	4	2/5
12	0.25	5.50	5	1/4
	0.025	<1.00	.	17/17
	0.0025	<1.00	.	8/8
	None	...	.	29/29

* Expressed in Logs. to the base of ten.
† ICR Mice.

that an oncolytic effect was produced after an inoculation of as small a dose as $10^{0.25}$ mouse LD_{50}. Although it is difficult to evaluate quantitatively the results of this experiment, because of the experimental error involved, the yield of Bunyamwera virus was highest from cells obtained from mice injected in the first series (32 millions of tumor cells) with small concentrations ($10^{1.9}$- or $10^{2.6}$) of Bunyamwera virus. The oncolytic effect seemed to be delayed in mice injected with 12 million tumor cells.

Effect of Tumor Cell Concentration

In these experiments the virus concentration was kept constant but the number of ascites cells in the inoculum varied. The results of such experiment with Ehrlich ascites cells and Bunyamwera and West Nile viruses are shown in table 3.

TABLE 3

Effect of Number of Malignant Cells in Inoculum Upon Susceptibility of Ehrlich Ascites Tumor to Oncolytic Action of Viruses

Virus	No. of Cells Inoc.*	Mouse LD_{50} Virus†	Mortality Ratio of Mice	Average Day of Death	Virus Titer‡ of Ascitic Fluid	Oncolysis Days After Virus Inoc.	Bioassay Tumor Ratio
	32	4.25			4.50	2	N.T.
		None			...	.	12/12
	16	4.25			5.80	2	0/2
		None			...	.	12/12
Bunyamwera							
	8	4.25	All mice died of virus		5.50	2	0/1
		None	infection		...	.	6/6
	4	4.25			6.50	1	0/3
		None			...	.	5/5
	18	4.8	3/6	29 ± 5.9			
		None	8/8	16 ± 2.3			
	9	4.8	1/6	13			
		None	6/6	15 ± 3.3			
	4.5	4.8	1/6	17			
		None	6/6	22 ± 4.0			
West Nile‡							
	2.25	4.8	0/6	..			
		None	5/6	19 ± 2.3			
	1.125	4.8	0/6	..			
		None	5/6	14 ± 0.8			

* x 10^6.
† Expressed in Logs. to the base of ten.
‡ PRI Mice.

After inoculation of $10^{4.25}$ mouse LD_{50} of Bunyamwera virus into tumor bearing mice, the yield of the virus was highest from mice which received one day previously 4×10^6 cells. The results obtained with West Nile virus were more uniform and protection of virus-infected mice was noted in all instances, except for partial oncolysis when the highest concentration of tumor cells was used.

The Krebs 2B is the most virulent tumor ever produced in ascites form (4). The effect of West Nile and Louping ill viruses is shown in table 4. The results had to be evaluated on the basis

TABLE 4

Effect of Number of Cells in Inoculum Upon Susceptibility of Krebs 2B Ascites Tumor to Oncolytic Action of Viruses

Tumor Inoculum No. of Cells x 10^6	Virus		Mortality of Mice* Per Cent	Average Day of Death	Oncolysis Days After‡ Virus Inoc.
	Type	LD_{50}			
5	West Nile	4.15†	100	30 ± 5.5	9
		3.15	70	42 ± 2.7	17
	Louping ill	5.20	80	36 ± 6.7	19
	Controls		100	12 ± 6.7	..
2.5	West Nile	4.15	88	28 ± 5.5	9
		3.15	90	41 ± 5.7	10
	Louping ill	5.20	50	59 ± 25.2	17
	Controls		100	9 ± 0.6	..

* PRl strain of mice.
† Expressed in Logs. to the base of ten.
‡ As indicated by microscopic examination.

of the average day of death rather than on percentage basis of surviving mice. Both viruses showed protective effect and no marked differences were noted in relation to the concentration of malignant cells in the inoculum.

A more quantitative investigation of the same problem is presented in table 5. In these two experiments decreasing concentrations of Krebs 2B tumor cells were implanted in PR1 mice which, either one or two days later, received the West Nile virus. Again, because of the highly invasive character of the tumor, the results had to be evaluated by the average day of death of the virus-treated and control animals. Using this criterion, the best effect in both experiments was observed in mice which received the smallest concentration of tumor cells. The average day of death was greater when virus treatment was started two days after implantation of the tumor. These results, however, again may reflect experimental error unavoidably present in such types of experiments.

Effect of Delayed Administration of Virus After Implantation of Tumor

In all experiments, results of which were summarized in the preceding section, the virus was administered four days after implantation of the Ehrlich ascites tumor cells and one or two days

TABLE 5

Effect of Number of Cells in Inoculum and Time of Virus Administration Upon Susceptibility of Krebs 2B Ascites Tumor to Oncolytic Action of West Nile Virus

Tumor Inoculum No. of Cells x 10^6	Virus Inoculum: Mouse LD_{50}	Virus Inoculum: Days After Tumor Implan.	Mortality Ratio of Mice*	Average Day of Death
16	5.15	1	5/5	5 ± 0.3
	None	.	5/5	4.4 ± 0.4
8	5.15	1	4/5†	34 ± 8.7
	None	.	5/5	8 ± 0.8
4	5.15	1	4/5†	29 ± 11.5
	None	.	5/5	7 ± 0.6
8	4.85	1	6/6	15 ± 3.5
	4.85	2	6/6	7 ± 0.6
	None	.	6/6	7 ± 1.1
4	4.85	1	5/5	17 ± 4.3
	4.85	2	3/6	11 ± 6.5
	None	.	6/6	9 ± 1.1
2	4.85	1	6/6	22 ± 5.1
	4.85	2	5/6	31 ± 7.4
	None	.	6/6	10 ± 6.8
1	4.85	1	6/6†	23 ± 7.8
	4.85	2	5/6†	38 ± 7.3
	None	.	6/6	11 ± 10.4

* PRI Mice.
† Ascites free mice dying with massive growth of tumor in subcutaneous tissue.

after implantation of Krebs 2B tumor cells. It was of interest to determine whether the oncolytic effect would still occur when administration of the virus was delayed.

Groups of ICR mice were implanted with 5×10^6 cells of Ehrlich ascites tumor and then at intervals, as stated in table 6, injected intraperitoneally with Bunyamwera virus ($10^{5.0}$ mouse LD_{50}). The results of the two experiments A and B were similar and indicated that the oncolytic effect of the virus was still present even when the infection was introduced 12 days after tumor implantation, at a time when about 40 per cent of the non-infected animals were dying of malignancy. First signs of oncolysis were observed in mice which received the virus 4 and 6 days after tumor implantation, but even after a longer interval (12 days) oncolysis already occurred on the 5th day after the virus was introduced.

Route of Inoculation of Virus

The greater affinity of the virus for tumor cells, when inoculated intraperitoneally, was quite apparent from the results

TABLE 6

*Oncolytic Effect of Bunyamwera Virus Administered at Different Time Intervals After Implantation of Ehrlich Ascites**

Time Interval After Tumor Implantation Days	Exp.	Oncolysis Days After Virus Inoculation	Bioassay Tumor Ratio	
			Virus	Controls
4	A	2	0/1	5/5
6	A	3	1/5	6/6
	B	3	0/4	5/6
8	A	4	0/3	6/6
	B	7	0/3	6/6
10	A	4	0/3	6/6
	B	5	0/5	7/8
12†	B	5	0/6	3/5

* 5 x 10^6 cells used for implant.
† About 40 per cent of non-infected mice were dying from ascites tumor.

mentioned above. To determine the effectiveness of other routes of administration, Bunyamwera and West Nile viruses were injected subcutaneously and intravenously into mice implanted 4 days previously with Ehrlich ascites tumor. Because infection with the Bunyamwera virus causes death, evaluation of results had to be based on bioassay and microscopic examination of infected ascites fluid.

Although the actual titer of the infected ascites fluid in the second experiment was lower following the intravenous and subcutaneous injections of Bunyamwera virus, the oncolytic effect was the same as obtained in intraperitoneally injected mice, as indicated by microscopic examination of the ascites fluid and the results of bioassay (Table 7).

The infection of tumor-bearing mice with West Nile virus by subcutaneous route did not lead to oncolysis (Table 7). All of the mice injected with the virus intravenously died of malignancy but the average day of death was prolonged in comparison with the control group. In contrast only one out of eight mice injected with the virus by intraperitoneal route died.

Enhancement of Susceptibility to Viruses

Experiments described in the preceding sections were conducted with lines of transplantable mouse ascites tumor which

TABLE 7

Administration of Virus to Ascites-Bearing Mice by Various Routes

Virus	Exp.	Route	Mortality Ratio of Mice	Average Day of Death	Virus Titer Ascitic Fluid	Oncolysis Days After Virus Inoc.	Bioassay Tumor Ratio
		IP			5.75	3	0/3
	1†	IV			5.50	5	0/7
		SC			NT	6	NT
		Controls				None	6/6
		IP			5.75	3	0/4
Bunyamwera‡	2†	IV			1.75	3	0/4
		SC			1.50	3	0/6
		Controls				None	16/16
		IP	1/8*	25	NT		NT
West Nile‡	1†	IV	5/5	32 ± 8.2	NT		NT
		SC	7/8	19 ± 5.1	NT		NT
		Controls	8/8	23 ± 2.9	NT		NT

IP = intraperitoneal IV = intravenous SC = subcutaneous NT = not tested
* PRI strain of mice used; action of virus evaluated from survival of the original host.
† 12 million cells inoculated.
‡ 5 per cent mouse brain suspension used as inoculum.

were found to be highly susceptible to virus infection. It was of interest to determine whether mammalian tissue during the process of carcinogenesis becomes more susceptible to virus infection. The results of experiments dealing with the above problem have been described elsewhere (5) and only a brief mention will be made here.

The main objective of the study was to determine whether enhancement of susceptibility to viruses would occur during the development of cervical carcinoma in C3H/HE mice induced by repeated applications of 3-4 Benzpyrene intravaginally. Exfoliated cells from vaginal aspirates were screened for selected criteria of malignancy during the entire period of carcinogenesis. The correlation of cytologic records with diagnosis of uterine lesions in mice sacrificed at various stages of carcinogenesis permitted the recognition of cytologic patterns corresponding to a) non-neoplastic lesions, b) early carcinoma, and c) advanced carcinoma.

Groups of mice treated with 3-4 Benzypyrene or Croton oil (as control non-carcinogenic irritant) and a control untreated group were subjected to intravaginal paintings with Mengo, Bunyamwera and vesicular stomatitis viruses and the multiplication of the virus was determined by its presence in vaginal aspirates.

Susceptibility to virus infection was found to be significantly enhanced in the presence of neoplastic lesions. For instance, introduction of vesicular stomatitis virus intravaginally into mice subjected to an application of Benzypyrene for 15 weeks (Table 8) led to a local asymptomatic infection, which was not observed in mice that received the Croton oil application or in those that were untreated.

In another series of experiments along the same line, vaginal mucosa of 34 mice was painted repeatedly during 19 weeks with 3-4 Benzpyrene, Tween 60 and combination of the two. Tween 60 was used because of its alleged co-carcinogenic effect. Mice were then infected with the vasicular stomatitis viruses (Indiana and New Jersey strains) by insertion of infected cotton swab applicators into the vaginas. Vaginal aspirates were collected at time intervals shown in tables 9 and 10. It may be observed that the virus was recovered from vaginal aspirates of mice which were subjected to carcinogenic treatments with Benzpyrene alone, or in combination with Tween 60. Tissues of mice treated

TABLE 8

Comparative Effect of Carcinogen and Irritant Upon Susceptibility of Mice to Intravaginal Infection with Vesicular Stomatitis Virus (New Jersey Strain)

Pretreatment	Infectivity of Vaginal Aspirates Days After Virus Treatment		
	3	6	10
Benzpyrene*	+	+	+
Croton Oil*	0	0	0
None	0	0	0

* Treatment for 15 weeks.

TABLE 9

Presence of Vesicular Stomatitis Virus (Indiana) in Vaginal Aspirates of Mice Submitted to Carcinogenic Treatment

Carcinogenic Treatment	Infectivity of Vaginal Aspirates Days After Virus Treatment			
	2	5	9	12
Benzpyrene*	+	+	+	0
Benzpyrene and Tween 60*	+	+	+	0
Tween 60*	0	0		
None	0	0		

* Treatment for 19 weeks.

TABLE 10

Presence of Vesicular Stomatitis Virus (New Jersey) in Vaginal Aspirates of Mice Submitted to Carcinogenic Treatment

Carcinogenic Treatment	Infectivity of Vaginal Aspirates Days After Virus Treatment		
	3	5	7
Benzpyrene*	+	+	+
Benzpyrene + Tween 60*...	0	+	+
Tween 60*	0	0	0
None	0	0	0

* Treatment for 19 weeks.

TABLE 11

Correlation of Carcinogenesis and Susceptibility to Virus Infection Based on Cytologic Findings

Months of Benzpyrene Treatments	Number of Treatments	Ratio of Virus Infected Mice	Mice Showing Presence of Antibodies	No. of Mice With Smears Suggestive of Early Carcinogenesis at Time of Benzpyrene Treatment
1	8	2/25	1/5	0/20
2	16	1/25	0/6	0/20
3	24	3/24	0/5	4/20
4	32	14/25	11/12	9/20

with Tween 60 alone, or of the untreated controls, failed apparently to support growth of the virus.

The time at which the susceptibility of genital tissues of mice to virus infection was enhanced appeared to be related to the presence of early neoplastic lesions. This was substantiated in a study in which groups of C3H mice were treated with Benzpyrene for 8, 16, 24 and 32 times during 1, 2, 3 and 4 months respectively, and then infected intravaginally with the vesicular stomatitis virus (Table 11). The virus multiplied in tissues of mice after they were subjected to 32 treatments during 4 months, but not for a shorter period of time. Vaginal aspirates of about one-half of the mice after 4 months' treatment with Benzpyrene showed microscopically demonstrable presence of an early neoplasia. In other mice presence of early noeplastic lesions could be surmised because of multiplication of the virus, which failed to do so in mice not subjected to carcinogenic treatment.

In experiments with Mengo virus, the results indicate a similar pattern. As shown in table 12 the virus did multiply in the Benzpyrene treated mice, in contrast to the controls in which only 6 out of 56 mice yielded virus-infected vaginal aspirates.

In the course of these experiments the progress of a neoplastic process, which ordinarily ends in uterine cancer, was thoroughly followed and it was found that neoplasia was not halted by viral infection.

Another aspect on the enhancement of susceptibility of tissue to virus infection was studied in relation to tumors hitherto found to be resistant to the effect of viruses (see Figs. 1 and 2). In the course of a study reported in detail elsewhere (6) five ascites tumors, three mouse-grown (6C3HED, dba lymphoma and P 288) and two rat-grown (AH 130 hepatoma and Yoshida sarcoma) were adapted to progressive growth in hitherto insusceptible and incompatible host, the ICR stock of Swiss mice. As indicated previously (Fig. 1 and ref. 2) the original host specific tumors were resistant to virus infection, in marked contrast to the derived tumor lines which were found to be highly susceptible to the effect of virus multiplication.

Table 13 summarizes the degree of multiplication and the extent of oncolytic action of three viruses in relation to the tumors. It will be observed that Mengo and Bunyamwera viruses have undergone limited degree of multiplication in 6C3HED and dba lymphoma original tumors without showing any oncolytic action. In no other instance had any of the three viruses multiplied in the original lines of the ascites tumors. In contrast, the adapted lines not only supported growth of the virus but a marked oncolytic effect was observed. Even more remarkable results were obtained when Western equine encephalomyelitis and

TABLE 12

Isolation of Mengo Virus From Vaginal Aspirates of Benzpyrene-Treated Mice

Duration of Carcinogenic Treatment (Months)	Number of Virus Applications	Presence of Malignant Cells	Virus Isolation Ratio of Mice	
			B.P. Treated	Controls
5	8	+	6/6	0/6
7	12	+	10/25	5/25
2½	13	+	4/25	1/25
Total			20/56	6/56

Semliki Forest viruses were used to infect tumor bearing mice. These viruses were found to infect rarely, if at all, any of the transplantable tumors mentioned in figures 1 and 2. As shown in table 14, these viruses did multiply in all of the three derived tumor lines, infection leading to complete oncolysis. In marked contrast, none of the original tumors and Ehrlich ascites tumor supported growth of the viruses.

The same observations were found to be true for the original (rat-grown) and derived mouse adapted Yoshida tumor (Table 15). With the exception of Anopheles A virus, all of the other viruses caused oncolysis of the derived tumor, whereas none showed such an effect on the original tumor.

TABLE 13

Comparative Susceptibility of the Original and Derived Sublines of Ascites Tumors to the Effect of Viral Agents

Tumor	Mengo Mult.	Mengo Oncolysis	Bunyamwera Mult.	Bunyamwera Oncolysis	West Nile Mult.	West Nile Oncolysis
6C3HED						
Original	±	—	±	—	NIL	
Derived	+	+	+	+	+	+
DBA Lymphoma						
Original	±	—	±	—	NIL	
Derived	+	+	+	+	+	+
AH 130						
Rat	NIL		NIL		NIL	
Mouse	+	+	+	+	+	+

TABLE 14

Effect of Infection with Semliki Forest and Western Equine Encephalomyelitis Viruses Upon Original and Derived Sublimes of Tumors

Tumor	Semliki Forest Virus: Log. LD_{50} Titer of Virus*	Semliki Forest Virus: Oncolysis	Western Equine Encephalomyelitis Virus: Log. LD_{50} Titer of Virus*	Western Equine Encephalomyelitis Virus: Oncolysis
6C3HED				
Original	2.5	—	<UND.	—
Derived	6.0	+	5.0	+
DBA				
Original	1.0	—	<UND.	—
Derived	5.5	+	6.5	+
AH 130				
Rat	<UND.	—	<UND.	—
Mouse	4.2	+	6.5	+
Ehrlich	1.5	—		

* In the ascites fluid, 3–4 days after inoculation of virus.

TABLE 15

Susceptibility of Original and Derived Yoshida Ascites Tumors To Infection With Viruses

Virus	Tumor: Original — Log. LD_{50} Titer of Virus*	Original — Oncolysis	Derived — Log. LD_{50} Titer of Virus*	Derived — Oncolysis
Mengo	1.5	—	8.0	+
Bunyamwera	2.5	—	4.5	+
West Nile	<0.5	—	1.5	+†
Semliki Forest	<2.0	—	>7.5	+
Western Equine Enceph.	<0.5	—	4.5	+
Eastern Equine Enceph.	<0.5	—	>6.5	+
Vesicular Stomatitis	<0.5	—	>7.5	+
Anopheles A	<0.5	—	3.5	—
Anopheles B	<0.5	—	4.5	±

* Of ascites fluid.
† Experiment in PR mice. All recovered from tumor.

Release of Antigenic Material from Virus Infected Ascites Tumors

The rapid and effective destruction of the ascites tumor at the height of its growth by virus infection (see Table 6) led to investigations in which infection with West Nile virus was introduced into tumor bearing PR1 mice. This particular system was chosen because of the resistance of PR1 mice to the lethal effect of West Nile virus (see Tables 1 and 3). After the destruction of the tumor by virus was achieved, mice were "challenged" with isologous and homologous tumor lines. Originally the purpose of this study was to detect antigenic relationship between the derived and original tumor lines, and among the derived lines themselves. Later this method was applied to a general investigation on antigenic relationship between different ascites tumors to which PR1 mice were susceptible.

As shown in table 16, mice which recovered from malignancy induced by the derived line of the dba lymphoma after West Nile infection, received 17 days later another implant of the same tumor. Six out of ten resisted completely the implantation and were 14 and 28 days later challenged with dba-original and 6C3HED-original tumors by subcutaneous implantation. In nontreated PR1 mice massive subcutaneous implants of these incompatible tumors will lead to development of a subcutaneous tumor

which ultimately regresses, as shown in the last column of table 16. The tumor failed to grow completely in the mice previously immunized by the derived line of dba lymphoma.

The same type of procedure was used to study the antigenic relationship between the AH 130 mouse adapted derived line and three other derived tumors, mentioned in table 17. Here, following recovery from the original tumor implant after West Nile infection, and verification of immunity by resistance to second implant of the same tumor, mice were divided into three groups and "challenged" with three other derived tumor lines. As indicated by the high survival ratio of test mice (Table 17), compared to the almost uniform mortality rate of untreated control mice, the antigenic material released from AH 130 hepatoma by the virus infection was highly effective in conferring immunity against at least three other tumor lines.

In experiments summarized in table 18 the mouse adapted derived line of Yoshida ascites tumor was used as primary inocu-

TABLE 16

Immunological Relationship Between DBA Derived Line and Other Ascites Tumors

Exp.* No.	2nd Inoc. (I.P.)	3rd Inoc. (I.P.)	4th Inoc. (Subcut.)	Days Between	Ratio of Mice Dead of Tumor	
					Test	Control
1	West Nile			7	2/12	1/1
		DBA Der.		14	4/10	5/5
			DBA Orig.	14	0/3	3/3†
			6C3HED Orig.	28	0/2	3/3†

* First inoculum prior to virus 10^6 cells of DBA derived tumor.
† Subcutaneous tumors ultimately regressed.

TABLE 17

Immunological Relationship Between AH 130 Derived Line and Other Ascites Tumors

Exp.* No.	2nd Inoc.	3rd Inoc.	4th Inoc.	Days Between	Ratio of Mice Dead of Tumor	
					Test	Control
1	West Nile			7	2/18	2/2
		AH 130 Der.		14	1/16	2/2
			DBA Der.	14	1/5	2/3
			Yoshida Der.	14	0/2	5/5
			6C3HED Der.	39	0/3	2/2

* First inoculum prior to virus 10^6 cells of AH 130 derived tumor.

TABLE 18

Immunological Relationship Between Yoshida Derived Line and Other Ascites Tumors

Exp.* No.	2nd Inoc.	3rd Inoc.	4th Inoc.	Days Between	Ratio of Mice Dead of Tumor	
					Test	Control
2	West Nile			7	1/18	2/2
		Yoshida Der.		14	0/15	2/3
			Yoshida Der.	14	0/5	5/5
			AH 130 Der.	14	0/3	3/3
			6C3HED Der.	24	0/5	2/2
			DBA Der.	14	3/5	2/3

* First inoculum prior to virus 10^6 cells of Yoshida derived tumor.

TABLE 19

Immunological Relationship Between Ehrlich 4n and Other Ascites Tumors

Exp.* No.	Virus	Tumor	Days Between	Ratio of Mice Dead of Tumor	
				Test	Control
1	West Nile		7		
		Ehrlich	14	1/6	3/3
		SA 37	14	5/6	3/3
		DBA Der.	14	2/6	2/3
		AH 130 Der.	14	1/4	3/3

* First inoculum prior to virus 10^6 cells of Ehrlich ascites tumor.

lum. The resistance of the 15 mice to challenge with the same tumors was complete. The re-challenge of these mice with the same and three other derived tumor lines indicated resistance to the AH 130 and 6C3HED derived ascites tumor but not to the derived dba lymphoma.

When Ehrlich ascites tumor was used as primary inoculum (Table 19) the recovered mice were found to resist the dba derived and the AH 130 derived tumors, but not the Sarcoma 37 ascites tumor. When, however, AH 130 or dba lymphoma derived lines were used for immunization purposes, a definite resistance to Sarcoma 37 was also conferred.

Effect of Release of Antigenic Material by Viruses Upon Spontaneous Tumors of Mice: Cancer Cure of 1957

The extremely powerful antigenic effect of virus-destroyed ascites tumor cells tempted the author to try a similar experi-

ment in mice bearing spontaneously developed tumors. Unfortunately, the incidence of spontaneous tumors in the PR1 strain of mice is almost nil, and the experiment had to be conducted in either ICR or C3H/HE tumor bearing mice. The results of two such experiments are shown in figures 3 and 4. In both cases tumors were carefully palpated, their size measured, and then the derived line of Yoshida ascites tumor was implanted intra-abdominally, followed 4 or 7 days later by West Nile virus injected by the same route (see Figs. 3 and 4). Control mice received only West Nile virus without previous ascites tumor implantation. The size of tumors was determined every two days,

FIG. 3

Effect of West Nile Virus Infection of Ascites Tumors Implanted into C3H/He and ICR Mice Bearing Spontaneous Tumors

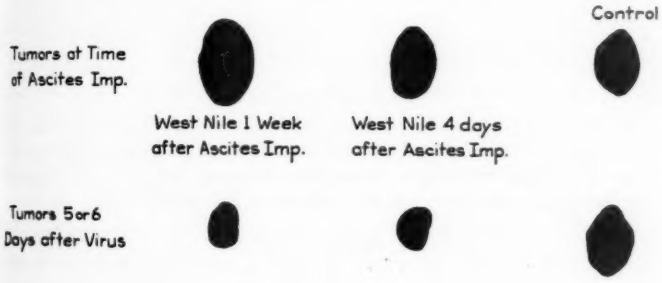
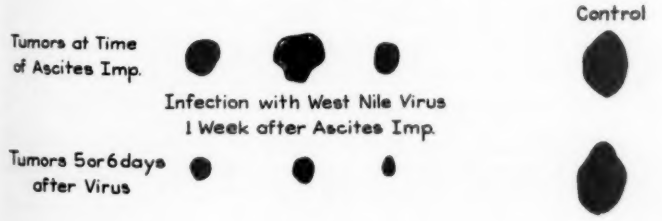

Control
Tumors at Time of Ascites Imp.
West Nile 1 Week after Ascites Imp.
West Nile 4 days after Ascites Imp.
Tumors 5or6 Days after Virus

FIG. 4

Effect of West Nile Virus Infection of Ascites Tumors Implanted into C3H/He and ICR Mice Bearing Spontaneous Tumors


Control
Tumors at Time of Ascites Imp.
Infection with West Nile Virus 1 Week after Ascites Imp.
Tumors 5or6days after Virus

but the experiment had to be terminated 7–9 days after virus inoculation since all of the mice died from West Nile virus encephalitis.

In the first experiment (Fig. 3) test mice bore larger spontaneous tumors than in the second experiment (Fig. 4). The destruction of induced ascites tumor cells by the West Nile virus, as verified by microscopical observation of ascites fluids, seemed to influence adversely growth of the spontaneous tumor. In 5 mice tested, a decrease in tumor size seemed to be noticeable. Infection with West Nile virus alone, without previous implantation of tumor, did not have any effect upon spontaneous tumors.

Because of death of the animals from the virus infection, these experiments cannot be classified as even remotely conclusive. It is quite possible when a greater number of animals will be used, these preliminary results will not be confirmed. Thus, the opportunity to present "The Cancer Cure (of mice) of Year 1957" may not arise again. This would justify the definition of opportunity by Ambrose Bierce as "a favorable occasion for grasping a disappointment"(7).

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STABLE AND PURE LINE CELL STRAINS: DERIVATION AND COMPARATIVE VIRAL SUSCEPTIBILITY*

JEROME T. SYVERTON AND LEROY C. MCLAREN†

The animal cell and virus increasingly are the focus for converging interest of biochemists, geneticists, cancer biologists, and virologists. Poliomyelitis research itself has been responsible for revolutionary advances in cell culture methodology and studies of animal cell biology and biochemistry. The result has been a succession of new problems so rapidly presented as to leave little time for thorough exploration of their consequences. One fact is clear: the virus is a sufficiently useful key to the inner workings of cells that trails hacked out in search of poliovaccine may well become roads to control of cancer.

In particular, the quest for new cultivable cells to serve as agents for detection and production of viruses has quickly made available a broad spectrum of animal cell types for *in vitro* study. Distinction and classification of the properties and behavior of these cells will be generally valuable. The finding, then, that cells in culture vary in their response to viruses makes viral susceptibility a useful criterion for differentiation of cells. Our department has a dual interest in viruses (both virulent and tumorigenic) and cancer, which has led us to maintain and derive a wide variety of human cells in culture. These cells are being studied with respect to viral susceptibility, growth and metabolic behavior, response to radiation, chromosome pattern, and capacity for heterologous transplantation (Syverton, 1957).

The present report describes the derivation of some new human cell strains, showing the relatively simple principles involved, and gives an account of some studies on comparative viral susceptibility. The latter subject, taken up initially as a purely practical problem in virology, has wide implications.

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† Department of Bacteriology and Immunology, University of Minnesota, Minneapolis.

Derivation of Cell Strains

A few years ago the establishment of cells in continuous culture appeared quite difficult, and isolation of strains from single cells equally so. Recently Puck, Marcus, and Cieciura (1956) were able to circumvent difficulty encountered in the latter technique, first by plating single cells over a feeder layer of irradiated cells, then by eliminating the necessity for a feeder layer in most instances by minimal trypsinization and gentle handling of cells during dispersion. Although initial establishment of cells in continuous culture still cannot be called easy, benefiting from the experiences of Pomerat, Gey, Earle and other leaders in this field, we have had success with the following procedure.

General protocols for preparation of Hanks's solution (BSS) and maintenance solution (MS), preparation of chick embryonic extract (EE) and serum, enumeration of cells and production of cultures from dispersed cells in tubes or bottles, have been described (Syverton, Scherer, and Ellwood, 1954). In addition to the usual precaution of obtaining as free as possible from cholesterol, pretesting for toxicity to cells in culture served to select human sera taken from single donors for use in establishment of cell strains. The stock basal medium (YEM) for dilution of serum contains 10 parts of 1 per cent solution of Difco Yeastolate, 2.5 parts of 10 per cent glucose solution, and 87.5 parts BSS (Robertson *et al.*, 1955). When increased buffer capacity is desired, the BSS is fortified with 0.113 g. per cent of sodium acetate and 0.064 g. per cent of sodium pyruvate. Mixture of 20 parts human serum (HuS), 76 parts YEM and 4 parts 1.4 per cent sodium bicarbonate provides excellent growth medium. For maintenance of minimally metabolizing cells, 3 to 5 parts of human, chicken, monkey or calf serum are combined with 5 parts of 1.4 per cent sodium bicarbonate and 90 parts YEM. Penicillin and streptomycin are added to all media in respective concentrations of 100 units and 100 micrograms per ml.

Cells in primary culture are propagated from small pieces of tissue taken as specimens from patients admitted to University of Minnesota Hospitals for elective surgery. After reception, tissue is stored usually for no more than 1 hour in HuS-20, YEM-80 at 4° C. For explantation, tissue is wetted with a few drops of embryonic extract and cut with scalpel blades (manipulated

scissor-like) into fragments 0.5 to 1.5 mm. in diameter. Six explants in embryonic extract are aligned on a coverslip coated with a minimum amount of human or chicken plasma, and contained in a Leighton tube. After incubation, six to ten of these tube cultures commonly provide enough cells for passage in series. Each serial passage is initiated in duplicate with cells from two primary cultures; one set is started with cells detached from the parent culture with a rubber policeman and the other by cells removed with trypsin. The dual procedure guards against occasionally observed toxicity of the enzyme (Difco trypsin 1:250, 0.1–0.2 per cent in MS–100 at pH 7.4–7.6) for cells in primary culture. Cells are exposed to enzyme at 37° C. for 5 minutes or less, immediately suspended in cold YEM medium which halts tryptic action, and sedimented gently at speeds not exceeding 1,000 r.p.m. Cells are resuspended in growth medium to a concentration of at least 100,000 per ml. and transferred to new tubes or flasks, to achieve serial passage. After a year of such passage in continuous culture, cell strains are considered stable and established.

Clonal strains are derived from parent lines by three successive single-cell isolations by either of the two techniques of Puck, Marcus, and Cieciura (1956). By a third method, high dilutions of dispersed cells are plated in a thin layer of human or chicken plasma on glass, which with some cells encourages clonal growth.

Cells of established strains in continuous culture have been maintained in our laboratory since 1952. Methods of culture have not been changed since last description (Syverton, Scherer, and Ellwood, 1954) except for routine use of YEM medium. Six stable and numerous clonal lines of the *HeLa strain* of uterine cervical carcinoma, originally supplied by Dr. G. O. Gey in plasma-clot culture, are in general use. The fact that sublines of the cell have been sent by request to 183 laboratories in all parts of the world for mass production of cells emphasizes a situation analogous to that current with inbred mice. Subline differences observed in this laboratory and elsewhere indicate the necessity for designation and description of each subline. Two lines of the *Maben strain* of pulmonary adenocarcinoma, propagated through 30 or more passages since reception in December, 1955, from Dr. A. W. Frisch (1955), have been parent to several clonal lines. The *conjunctival cell* propagated by Chang

(1954) and described by McAllister and Coriell (1956) was obtained in March, 1955, from Dr. John Snyder. Thirteen months later, three clonal strains now more than 150 passages removed from the donor were derived. In December, 1955, and many times since, clones were propagated from Chang's (1954) *liver cell*, procured earlier in March from Dr. Smadel. Now 90 passages removed, multiple clonal lines exhibit unusual growth vigor and other interesting properties. Until routine use of HuS-20, YEM-80 growth medium, we shared the difficulties in cultivation and maintenance of the conjunctival and liver cells experienced by Chang (1954). The wide variety of natural and synthetic media rapidly being made available for cell cultures should, we believe, be cautiously applied to the derivation and maintenance of stock cultures until selective influences on culture constitution can be evaluated. This conservative attitude does not depreciate the value of these media for studies in virology and physiology.

Of seven cell strains originated from human surgical specimens from 13 patients between November, 1955, and October, 1956, three have been kept in continuous culture for more than a year and hence are considered established strains. Among strains surviving seven or less months, two derived from normal lung parenchyma were passed respectively four and eight times during a 3-month period. An esophageal fibrocyte grew vigorously through 21 serial passages, only abruptly to cease multiplication without apparent cause after more than 6 months in culture. Attempts to propagate cells from normal or malignant tissues of the gastrointestinal tract and bladder uniformly failed. More successfully, a human kidney epithelial cell has been kept slowly reproducing for 5 months. A slow-growing palate fibrocyte now has survived 29 transfers after more than 15 months of continuous culture. This fibrocytic cell is approximately a third the size of the esophageal fibrocyte, less vigorous, and slower in growth. A strain of vigorously growing esophageal epithelium is in its 32nd generation; this parental strain and clonal derivatives are now widely used in our laboratory. A third cell of remarkable vigor and reproductive capacity was derived from biopsied liver of a 15-month-old infant; 27 passages have been accomplished in less than 9 months. Successful establishment of these new human cell strains (as well as strains from

other animal species) we attribute to use of yeast extract medium and the thin plasma layer for primary culture and clonal isolation. A 6.4 cm. human fetus obtained in December, 1956, has yielded cell lines now in 3rd to 8th passage, representative of 5 tissues. Three of these lines, by their reproductive vigor, give reasonable assurance of attaining continuous culture.

Comparative Susceptibility of Stable and Clonal-Strain Epithelial Cells to Infection by Viruses of Poliomyelitis, Vaccinia or Herpes Simplex

Numerous laboratories are employing a variety of malignant and non-malignant cells for virus research, including cells in primary culture (as for example, those of monkey kidney), morphologically similar cells of established strains, and more recently cells of clonal origin. All of these show an all- or-none response to certain viruses. Particularly with polioviruses, it has also been reported (Kibrick, 1955; Wenner, 1955; Fogh and Lund, 1955) that the cells differ in respect to susceptibility or sensitivity to virus infection. This rather vaguely conceived property is defined by the term "plating efficiency" originated in work with bacterial viruses; that is, the capacity of one cell type, when used in assay of a single virus, to reflect a greater or lesser titer than another cell type is a measure of its efficiency or sensitivity as an assay host. Both extrinsic and intrinsic factors could affect this property of cells, or as it actually is, property of cell *cultures*. What has not been clear is whether differences in susceptibility of this type are determined by cellular factors or by conditions of culture. The wide variety of stable and clonal cell strains just discussed was applied to the problem, by determination of comparative titers given by all of the cells in assays of each of the polioviruses (Types 1–3), vaccinia and herpes simplex virus. Monkey kidney cells in primary culture were employed for comparison. All of the cells were propagated in YEM medium by uniform procedures standardized in our laboratory. Dispersed cells were dispensed into tubes for dilution titrations or into rectangular screw-capped bottles for plaque counts. For dilution titrations, the human serum growth medium was rinsed from tube cultures and replaced with 10 per cent calf serum in YEM for maintenance. Bottle cultures inoculated with virus for plaque counts were overlaid with an agar medium containing chicken

serum and YEM. Tables 1 and 2 show results of dilution titrations of the five viruses with cells of parental and clonal strains and monkey kidney cells. The two sets of titrations were done with independently prepared virus pools; parental HeLa and clonal A_1 liver cells were included in both sets as controls. In this laboratory replicate titrations give titers (expressed as $TCID_{50}$) differing at most by ± 0.3 log. Examination of the titer ranges, appended at the bottom of each table, for all cells against each virus shows that no great differences in comparative susceptibility were observed. Only differences of the same order were found: a) in titrations of the five viruses with cells of four clonal

TABLE 1

Comparative Titers of Herpes, Vaccinia and Polioviruses (Pool B) by Dilution Method With Tube-Cultures of Epithelial Cells

Cell Strain	Titer as Log. $TCID_{50}$ Per ml. for				
	Poliovirus Type 1 Mahoney	Poliovirus Type 2 MEF–1	Poliovirus Type 3 Saukett	Herpes Simplex O'Connell	Vaccinia 5 Per Cent CAM
HeLa, parental	7.8	7.7	6.8	5.6	6.2
Maben, parental	8.0	8.0	7.0	5.8	6.6
Liver, parental	7.7	7.7	7.2	5.7	6.2
Liver, clonal A_1	7.7	7.6	6.9	6.4	6.2
Conjunctiva, parental	7.9	7.8	7.1	6.1	6.2
Esophagus, parental	7.9	8.0	7.3	5.8	6.0
Monkey kidney	8.2	8.1	7.2	5.7	6.2
Titer range, log $TCID_{50}$	0.5	0.5	0.5	0.8	0.6

TABLE 2

Comparative Titers of Herpes, Vaccinia and Polioviruses (Pool A) by Dilution Method With Tube-Cultures of Epithelial Cells

Cell Strain	Titer as Log. $TCID_{50}$ Per ml. for				
	Poliovirus Type 1 Mahoney	Poliovirus Type 2 MEF–1	Poliovirus Type 3 Saukett	Herpes Simplex O'Connell	Vaccinia 5 Per Cent CAM
HeLa, parental	7.5	7.3	6.8	4.8	5.3
HeLa, clonal 3	7.5	7.3	6.8	4.8	4.8
Maben, clonal 3	7.3	7.2	6.6	4.4	4.9
Liver, clonal A_1	7.5	7.2	6.7	4.8	5.5
Liver, clonal A_2b	7.5	7.4	6.7	4.2	5.3
Conjunctiva, clonal B_2A_4	7.3	7.1	6.7	4.2	5.2
Esophagus, clonal A_2b	7.7	7.6	6.7	4.2	5.2
Titer range, log $TCID_{50}$	0.4	0.5	0.2	0.6	0.7

sublines derived during 40 successive generations of parental liver epithelial cells; or b) when each of the five viruses was adapted to liver or esophagus epithelial cells by serial passage amounting to a cumulative dilution of original material of 10^{-18}, and titrated with homologous cells or those of HeLa or monkey kidney. A more quantitative measure (plating efficiency) of cellular susceptibility was provided by comparative plaque counts of each of the five viruses with cells of the type or strain listed in table 3. It is evident that monkey kidney cells and cells of all human strains tested, with the possible exception of Maben strains, were about equally sensitive to the viruses. Investigation of the quantitative bases of the somewhat greater efficiency of Maben cells suggested here is underway; the difference if substantiated can be a useful tool for studying Maben cells. Factors influencing differential cellular susceptibility to viruses, such as viral heterogeneity, cellular heterogeneity, host cell physiology, quantitative relations between virus and cells, and cellular yield of virus, are of major interest to virologists for reasons of both theory and practice. For the cell culturist, these relations define important capacities and properties of the cells themselves. The point to be made at present from these findings in contrast to the experience of others is that cells differing widely in type

TABLE 3

Comparative Plaque Counts of Polioviruses With Bottle Cultures of Epithelial Cells

Assay Cell Strain	Plaque Counts of Poliovirus Types 1 (Mahoney) Average 10^6 PFU/ml.	1 (Mahoney) Log. PFU/ml.†	2 (MEF–1) Average 10^6 PFU/ml.	2 (MEF–1) Log. PFU/ml.†	3 (Saukett) Average 10^6 PFU/ml.	3 (Saukett) Log. PFU/ml.†
EE*, parental	20	7.4 ± 0.4	32	7.5 ± 0.2	3.8	6.6 ± 0.1
EE, clonal	45	7.7 ± 0.2	67	7.8 ± 0.1	3.2	6.5 ± 0.1
Liver, parental ..	41	7.6 ± 0.1	38	7.6 ± 0.1	3.8	6.6 ± 0.4
Liver, clonal A_1A_1	28	7.4 ± 0.1	38	7.6 ± 0.1	3.6	6.6 ± 0.1
Conjunctiva, clonal	20	7.3 ± 0.1	14	7.1 ± 0.2	2.8	6.4 ± 0.1
Maben, parental..	160	8.2 ± 0.2	160	8.2 ± 0.2	8.2	6.9 ± 0.3
Maben, clonal ...	25	7.4 ± 0.2	100	7.9 ± 0.1	6.0	6.8 ± 0.1
HeLa, parental ..	29	7.5 ± 0.4	43	7.6 ± 0.4	4.1	6.6 ± 0.2
Monkey kidney ..	56	7.8 ± 0.3	62	7.9 ± 0.3	5.8	6.8 ± 0.2
Count range, log PFU‡		0.5		0.8		0.4

* EE = esophagus epithelial cell strain (Syverton).

† Mean log for 2–6 independent counts, ± ½ range of counts. Each count determined as average result for duplicate bottle cultures.

‡ Range calculated for average counts obtained with each virus and all cells, except Maben strains.

and origin, but maintained in the same laboratory by the same staff using a single medium and standardized cultural procedure, show surprisingly little differential susceptibility to the viruses tested. Studies of other features of cellular behavior, pertaining to nutrition, physiology, or malignant state, should be carried on with due consideration for possible effects on source cultures of variation in even minor details of cultural procedure.

Summary

Widespread use throughout the world of some cell strains established in continuous culture, with probable separation of distinct sublines, gives reason for designation of strains by laboratory. The derivation and propagation of some cell strains maintained at the University of Minnesota are described: these include parental and clonal substrains of HeLa, Maben, human conjunctiva and human liver. Procedures for establishment of stable strains and isolation of clonal lines, based primarily upon use of yeast extract medium for propagation and plating of cells in thin plasma layers, are reported. Seven new cell strains were initiated from human surgical specimens from 13 patients. Three cell types, including a palate fibrocyte, esophageal epithelial cell, and liver epithelial cell have been established in continuous culture. Parental and clonal strains of esophageal epithelium are widely used in this laboratory for virus studies. A variety of established and clonal human cell strains, maintained in a single medium by standardized procedure, were surveyed for differences in sensitivity to viral infection comparable to those reported in the literature. No significant differences in comparative susceptibility to Types 1–3 poliovirus, vaccinia or herpes simplex virus were revealed by titrations with stationary tube cultures. In plaque counts of polioviruses, kidney cells in primary culture and all strains of human cells, with possible exception of a line of pulmonary adenocarcinoma cells, exhibited about equal plating efficiency. It was concluded that the 14 strains of human cells studied were equally efficacious when employed under conditions of rigid procedural standardization.

Discussion

Dr. T. C. Hsu: I certainly should like to thank Dr. Syverton for such a superb paper. We enjoyed hearing it. I have a few tech-

nical questions to ask Dr. Syverton about his establishing of cell strains. We understand he used YEM, which we also use following his recipe. We would like to know his vessels; does he use bottle cultures, or use roller tubes? Second, he mentioned about a fibrocyte strain which abruptly terminated in about 20 passages. Did he follow the chromosome counts for the previous generations before it died off? Is there not actually a small objection to using the strain name "EE" as "esophagus epithelium"? I think it is confused with our tissue culture terminology when we use it in "embryonic extract."

Dr. Syverton: The type of container employed consists of a small petri dish; exact dimensions are not critical but the one we use more commonly is the five centimeter. The only point of interest, perhaps, is that this sort of petri dish with terminal dilutions is put in a peanut butter jar. You have the right CO_2 arrangement and these cells do beautifully. The "EE," of course is laboratory jargon, and I quite agree we should not use it and should call it human epithelium.

Dr. Lennette: I, on the other hand was looking for the missing third "E" for "EEE," or Eastern Equine Encephalomyelitis.

Dr. Gey: You make the point that these cell lines are quite individualistic, and then you describe the susceptibility to some of the virus challengers, for example the Coxsackie as more or less non-specific. Don't you consider that maybe these differences are all wrapped in that seven-tenth of a log?

Dr. Syverton: I quite agree that the morphologic differences observed were not related to differences in virus susceptibility. Variation of 0.7 log is only 0.4 removed. Plaque counts confirmed these findings. The use of plaque counts was in a sense a disappointment since we had anticipated that the apparent difference of 0.5 log indicated by assay with six tubes per dilution would be established by use of the plaque technique. I feel reasonably certain that further virus studies will establish differences in cell characteristics.

Dr. Stanley: You undoubtedly have used human amnion cells, haven't you? You probably know that Dr. Fogh in our lab has found what he believes to be a significant difference between

monkey kidney and human amnion cell infection with polio virus. The human amnion in both primary and secondary culture gives significantly higher numbers of plaques. I would certainly recommend that you make use of the human amnion cells. I believe that there are indications from other laboratories in a screening program, where they are screening stools and respiratory extracts for viruses, that there have been many cases so far where they get viruses via the human amnion, which they cannot get in monkey kidney, Hela, or a variety of other cells. So I suspect that this indicated greater susceptibility may represent, in fact, a real difference. There was a question this morning having to do with the human amnion. We have processed about 400 of them in the laboratory during the past year. Of those, we think, there were only three or four where we got cells that were distinctly different, that were rounded up or granular. That is, usually you get what seems to be a fairly uniform type of cell. In the last few months Dr. Dunnebacke Dixon has been taking what was thought to be a uniform cell type and cloning it. She got quite a variety of cells from this and each of these cell types is now being carried in passage. We, too, are quite interested in the chromosome change because there is quite obviously a change taking place in these cells after eight or ten or a dozen passages. It seems to be in the direction of what people have said represents malignancy, whatever that may be.

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ONCOLYTIC PROPERTIES OF VIRUSES*

ALICE E. MOORE†

One of the practical reasons for studying the oncolytic properties of viruses is to find out if they have any possibilities for treatment of cancer patients. Such a program was begun under the clinical direction of Dr. Chester Southam (1951, 1952, 1954). The so-called exotic viruses were chosen for this study because our population had no antibodies against them, they were not known to cause disease, and they had shown some oncolytic ability against mouse tumors. The following viruses have been used: West Nile (four strains including Egypt 101), Ilhéus, Bunyamwera, and in a very small number of patients—dengue, Semliki, Newcastle Disease virus, mumps, vaccinia, and two other neurotropic viruses whose exact identity had not been established. Only the clinical findings of one of the West Nile viruses, Egypt 101, which had been recently isolated from the blood of a child in Egypt (Melnick *et al.*, 1951) will be presented. The patients chosen were those for whom no other method of treatment was available. The virus was usually administered intramuscularly, and its presence sought for thereafter by inoculating daily samples of blood intracerebrally into mice. Whenever possible serial biopsies were done, and the neoplastic, as well as whatever normal tissue obtained was examined for virus and histological change.

In a series of 84 patients who lived long enough for evaluation, 90 per cent developed an active infection as judged by a viremia persisting for two to three days. Nine, or 11 per cent, had symptoms of encephalitis; in seven it was transient and mild, but in two it was severe. Usually, however, the only symptom referable to the virus infection was a slight fever.

The results of the clinical studies are presented in table 1. In the first column the types of tumors are listed. In the second

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† Division of Experimental Pathology, Sloan-Kettering Institute for Cancer Research; and Sloan-Kettering Division, Cornell University Medical College, New York 21, N. Y.

TABLE 1

Localization and Effect of Egypt 101 on the Growth of Cancer in 84 Patients

Type of Cancer	Localization			Inhibition of Tumor		
	No	Yes	Selective	No	?	Yes
Carcinomas						
Epidermoid	3	5	5	14	1	0
Gastrointestinal	0	14	5	24	5	3
Other	1	4	1	8	0	0
Sarcomas						
Reticulum cell	0	5	1	2	2	3
Other	1	8	2	8	2	0
Total	5	36	14	56	10	6
Per cent	10	65	25	78	14	8

column the localization of the virus in tumor is indicated. Selective localization means that virus was present in greater quantity in the tumor than in blood or other tissue, that it persisted in the neoplasm longer, or that it was found only in the tumor. In the 55 patients from whom tumor could be obtained for examination, virus was found in 36 (65 per cent) and was selectively localized in tumor in 14 (25 per cent). The third column deals with the effect of the virus on tumor growth, and admittedly, evaluations of this type are difficult to make. Inhibition of tumor growth was measured by direct observation of diminution of tumor size by a number of observers or by evidence from x-ray studies.

Figure I shows a series of x-rays from a patient with reticulum cell sarcoma whose mediastinal mass shrank during the course of her virus infection. She died nonetheless. In some cases reversal of abnormal chemical findings gave supportive evidence for a beneficial effect. Histological studies turned out to be of little value because it was impossible to distinguish between the necrosis which might occur in the course of the disease and the necrosis produced by the virus (Newman and Southam, 1954). Of the 62 patients whose clinical course could be evaluated, 56 (77 per cent) showed no change, in 10 (14 per cent) there was a suggestive effect, and in 6 (8 per cent) there was definite tumor inhibition. It must be emphasized that the inhibiting effects were of a temporary nature and did not last long enough to be of real benefit to the patient.

A few incidental observations were made during the clinical study. The most favorable results were obtained with carcinomas

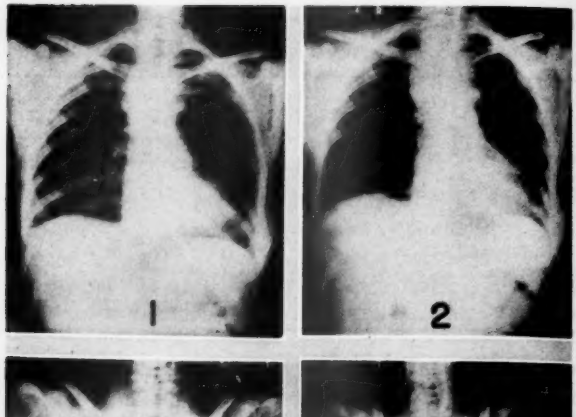


FIG. I. Serial x-rays of a 43 year old patient with reticular sarcoma inoculated with Egypt 101 virus. (1) Mediastinal mass, 7/3/52. (2) Increase in size of mediastinal mass by the day Egypt 101 was inoculated, 8/14/52. (3) Diminution in size of mediastinal mass by 9/19/52. (4) Further diminution in size of mediastinal mass by 10/9/52.

of the gastro-intestinal tract and the reticulum cell sarcomas. Although localization of virus was frequently obtained in the epidermoid cancers, no striking beneficial effects were noted. The clinical progress of five cases of cancer of the breast were also unaffected by the virus. It was also noted that the patients most susceptible to viral encephalitis appeared to be those with generalized involvement of the lymphatic system such as leukemia and reticular cell sarcomas.

Others also have investigated the clinical possibilities of viruses, and the older literature has been reviewed (Moore, 1954). More recently, Smith, Huebner *et al.* (1956) have reported the effects

of the APC viruses on thirty patients with epidermoid carcinoma of the cervix. The viruses, which ordinarily cause no disease, were introduced into the tumor by direct inoculation or by intra-aortic cathaterization. They noted an increase in the necrotic slough over that which would be normally expected in approximately 60 per cent of their patients. This effect seemed to be associated with the virus, since no slough was noted in six control patients. However, in all instances the tumor regrew, and at autopsy healthy growing cancer tissue could be detected at the rim of the lesion.

The clinical results in general can be summarized by stating that viruses do something to tumors sometimes but they are of no practical use now and will not be until all cancer cells composing a tumor are destroyed. That's a large order, but probably no larger than that which the chemotherapeutists face.

In the meantime investigations in the laboratory are being continued, and here the results are more encouraging. It is possible to completely destroy human tumors grown in tissue culture, on the chorioallantoic membrane of the eggs, and in the irradiated and cortisone-treated rat.

Fourteen viruses have been screened for destructive effects against a number of human cell lines grown in tissue culture. The cell lines used are HEP No. 1, No. 2, and No. 3 (all derived from human epidermoid cancers from the cervix, the laynx, and buccal mucosa respectively), HeLa, and a strain of human adult fibroblasts (HAF). Growth curves were done by exposing a 10^{-2} dilution of virus to the cells after they had grown on the side of the tube. The virus was removed after 1 hour at 37° C., the cell sheet was washed with balanced saline five times, and media added. Titrations were done one hour later, daily thereafter for four days, then on the seventh and tenth day. A tube inoculated with 10^{-2} virus but without cells was titered in the same way. Whenever a cytopathogenic effect was noted, a simultaneous intracerebral titration was done in mice and in the human cell line. Figures II–XV show the results for the fourteen viruses studied. The growth curves tell us quite a few things: 1) Most of these viruses grow in the tissue culture lines, some very well with the production of much virus, some poorly, and, of course, some not at all. 2) Most viruses have the same pattern of growth no matter what tissue culture cell line they are multiplying in.

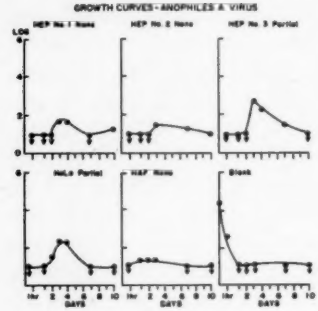
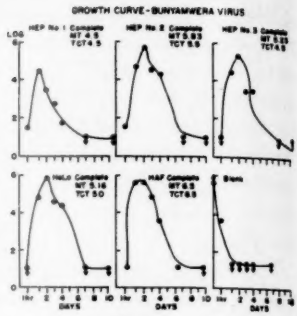


FIGURE II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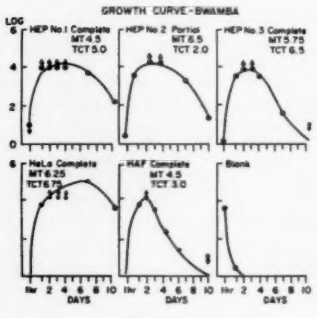
DAYS

FIGURE III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DAYS

FIGURE IV

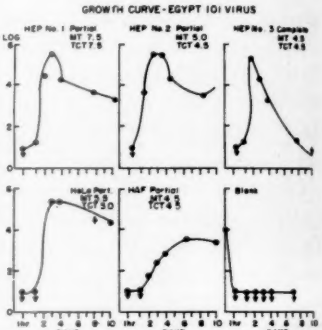
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FIGURE V

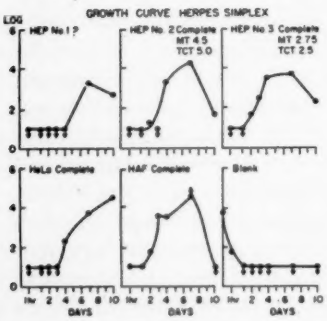
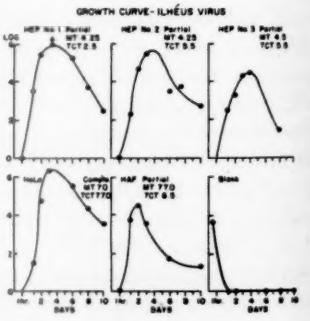


FIGURE VI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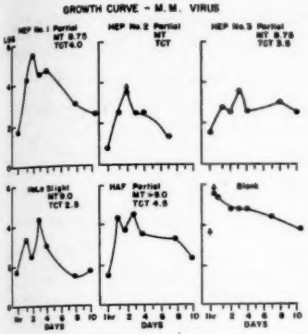
DAYS

FIGURE VII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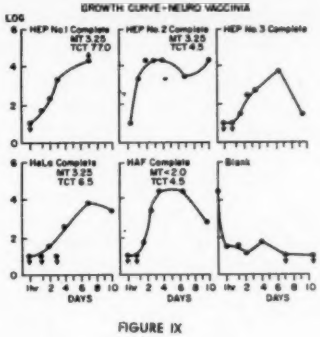
LOG
DAYS

FIGURE VIII


GROWTH CURVE - NEURO VACCINIA
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DAYS

FIGURE IX

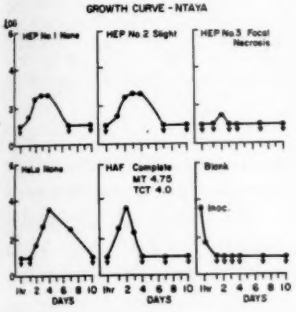
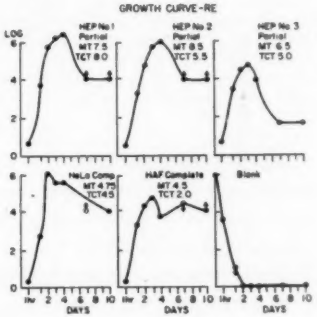


FIGURE X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DAYS

FIGURE XI

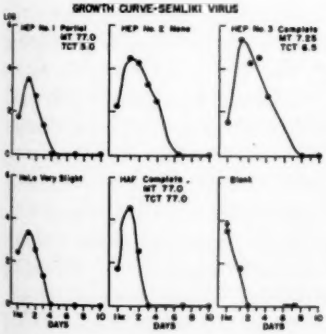

FIGURE XII

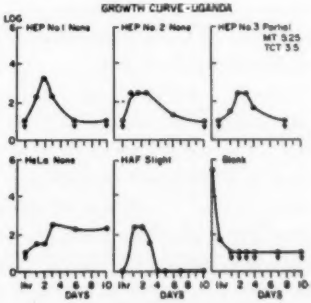

FIGURE XIII

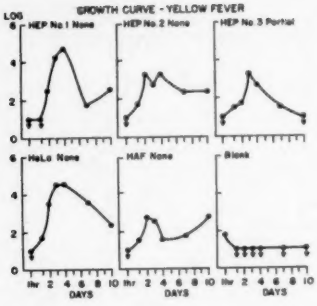

FIGURE XIV

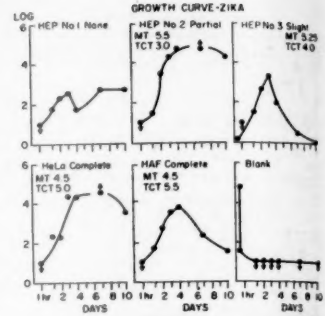

FIGURE XV

FIGS. II–XV. Growth curves for 14 viruses as follows: Anopheles A, Bunyamwera, Bwamba, Egypt, Herpes, Ilhéus, M.M., Nuerovaccinia, Ntaya, Russian encephalitis, Semliki, Uganda, Yellow fever, and Zika. Directly after the name of the cell line is the effect of the virus on the tumor; complete (++++), partial (++), slight (+), none (—). MT is the mouse titer expressed as the reciprocal of the LD_{50}. TCT is the tissue culture titer expressed as the reciprocal of the TCD_{50}.

3) There is no correlation between the growth of the virus and its destructive effect, as can be seen by the Semliki graph where as much virus was produced in the HEP No. 2 line, which was unaffected, as in the HEP No. 3 line, which was destroyed. 4) In all instances but one no cytopathogenic effect occurred without virus multiplication even though sometimes not much virus was produced, as, for example, with Anopheles A and HEP No. 3 tumor. The exception was Ntaya where areas of focal necrosis were demonstrated three times without the presence of any titratable virus. 5) In general the titer of the virus fell off after destruction of the host cells but this did not always occur as shown by the neurovaccinia and Russian encephalitis (RE) experiments. In some instances the virus disappeared even though the cells were not destroyed. This suggests that there may be auto-interference or perhaps virus inactivation at 37° C. had taken place. 6) It was observed that when the virus completely destroyed the cell line, the titer in mice and in tissue culture agreed within close limits. Even when destruction was partial as with Egypt, HEP No. 1, and HEP No. 2, there was good agreement between the two titrations. Most of the disparities noted were observed in the weakly oncolytic virus such as M. M.

Table 2 gives a summary of the oncolytic abilities of the cell lines and serves to emphasize the diversity of the results which are obtained. The RS No. 1 tumor is a rat sarcoma.

These same viruses were also tested against the HEP No. 3 tumor grown on the chorioallantoic membrane in fertile eggs by

TABLE 2

Tissue Culture Summary

Virus	Cell Line					
	HEP No. 1	HEP No. 2	HEP No. 3	HELA	HAF	RS No. 1
Anopheles A	—	—	++	++	—	—
Bunyamwera	++++	++++	++++	++++	++++	++++
Bwamba	++++	++	++++	++++	++++	—
Egypt	++	++	++++	++	++	—
Herpes	?	++++	++++	++++	++++	ND*
Ilhéus	++	++	++	++++	++	—
M.M.	++	++	++	+	++	—
Neurovaccinia ...	++++	++++	++++	++++	++++	++++
Ntaya	—	+	†	—	++++	—
Russian	++	++	++	++++	++++	—
Semliki	++	—	++++	+	++++	++++
Uganda	—	—	++	—	+	—
Yellow fever	—	—	++	—	—	ND*
Zika	—	++	+	++++	++++	—

* Not done.
† Focal necrosis.

a simple technique developed by Drs. Dagg and Karnofsky *et al.*, (1954). A piece of tumor about 2–3 mm. square is dropped on the chorioallantoic membrane of a nine-day embryo, and ten days later the tumors are harvested. The conditions of each test had to be varied according to the pathogenicity of the virus for the chick embryo when inoculated into the yolk sac. For example, if the virus killed rapidly, the eggs were not inoculated until the tumors had grown for three to five days. To determine the effect on tumor growth we have relied chiefly on bioassay experiments in which the virus-infected and control tumors are removed, weighed, minced, and a measured amount inoculated into another group of eggs (marked with an astrisk in Table 4) or into irradiated and cortisone-treated rats (Toolan, 1953) which were killed fourteen days later and their tumors were weighed. The tumor was also titered to determine the amount of virus present. Grading was done according to the scheme outlined in table 3. Table 4 gives the results of these experiments.

It can be seen that there is seldom any virus effect on the weight of the tumors, which perhaps should be expected because in many instances the experiments were terminated after three days incubation. In most instances the virus appeared in the tumor and attained high titers. This indicates some selection for tumor by virus, since it was inoculated into the yolk sac. The rat

TABLE 3

System of Grading Virus Effect on HEP No. 3 Grown on the Chorioallantoic Membrane

Grade	Initial Effect	Bioassay
++++	Too little time (1 day)	Complete inhibition
+++	Too little time (3 days)	Complete inhibition
++	None	Complete inhibition
+	None	Some inhibition
—	Same as control	Same as control

TABLE 4

Effects of Viruses on HEP No. 3 Grown on the Chorioallantoic Membrane

Virus	Initial Experiment T/I†	Av. Wt.	Tumor Titer	Bioassay T/I†	Av. Wt.	Grading
Anopheles A ...	4/5	0.34	+	9/9*	0.28	—
Control	7/9	0.22		8/9*	0.72	
Bunyamwera ...	12/13	0.04	5.5	0/4		+++
Control	15/15	0.2		6/8	2.18	
Bwamba	4/4	0.78	4.0	0/8		+++
Control	4/4	1.34		8/8	2.4	
Egypt	5/5	0.56	6.5	0/8		++
Control	9/11	0.66		8/8	5.42	
Herpes	3/4	0.83	>5.0	0/8*		++
Control	5/6	0.95		8/8*	0.38	
Ilhéus	9/10	0.37	6.0	5/8	0.17	+
Control	6/7	0.43		8/8	2.5	
M.M.	6/7	0.6	4.0	0/8		++
Control	11/11	0.94		6/8	0.63	
Ntaya	9/12	0.61	2.0	8/8	2.9	—
Control	9/13	0.58		4/4	6.7	
Russian E.	6/6	0.13	7.0	0/4		+++
Control	8/10	0.5		8/8	1.51	
Semliki	9/9‡	0.11	5.5	0/8		++++
Control	8/9	0.16		8/8	2.7	
Uganda	4/5	0.19	+	4/6*	0.04	+
Control	6/6	0.12		6/8*	0.44	
Yellow fever....	4/5	0.5	5.5	2/8*	0.11	+
Control	5/6	0.95		8/8*	0.36	
Zika	5/6	0.46	4.0	0/8		++
Control	11/11	0.48		8/8	3.45	

* Bioassay done in eggs.
† Number of tumors which grew/the number implanted.
‡ Bioassay done 24 hours after inoculation of 10 LD_{50}'s.

bioassay experiments show that of the thirteen viruses examined eight were capable of completely inhibiting tumor growth, 4 had very slight or no effect, and one was intermediate.

The testing of the viruses against HEP No. 3 grown in the irradiated and cortisone-treated rats was done as follows: Two million cells obtained from a trypsinized suspension of a solid tumor were inoculated subcutaneously into both flanks of groups of ten rats. When the tumors became palpable (usually in two to three days) 0.1 cc. of a 10 per cent brain suspension of virus was inoculated directly into the tumor. Similar controls were inoculated with 0.1 cc. of normal brain suspension. Ten days later the two animals bearing the largest tumors were killed and, in most instances, the others were left to observe the percentage of regressions. After weighing, a suspension of tumor cells was made, and treated rats were inoculated subcutaneously for bioassay. In each instance the cells were also inoculated into three different tubes to test the ability of the cells to grow in tissue culture, and titrations were done to determine the amount of virus present. Gradings were done as shown in table 5. Theoretically, it should be easy to determine if a virus destroys an implanted tumor in the rat simply by observing the percentage of regressions, but practically, it is not always easy. There may be a few spontaneous regressions among the controls, and the animals sometimes die of irradiation or cortisone before regressions take place. We therefore have depended on bioassay experiments. Table 6 gives our findings with thirteen viruses. The two most effective viruses were Bunyamwera and Egypt 101 which caused all of the tumors to regress with survival of the animals. Semliki was also quite effective, but it caused paralysis and death in most of the rats. Six of thirteen viruses had no effect at all on tumors, and the others (four) had a moderate inhibiting effect. Comparable results were obtained in tissue culture.

TABLE 5

Grading of Virus Effect on HEP No. 3 Grown in Prepared Rats

Grade	Initial Effect	Bioassay
++++	90–100 per cent regressions	No growth
+++	50 per cent regressions	No growth
++	None	No growth
+	None	Tumor wt. 1/5 of controls
—	None	Tumor wt. same as controls

TABLE 6

Effects of Virus on HEP No. 3 Grown in the Irradiated and Cortisonized Rat

Virus	Initial Experiment No. Regressions	Av. Wt.	Virus Titer	Bioassay T/I	Wt.	Virus	Grading
Anopheles A ...	2/10	2.8	<1.0	5/8	4.93	—	—
Control	2/10	3.26		8/8	2.89		
Bunyamwera ..	8/8	5.8	4.0	0/8			++++
Control	4/10	12.3		8/8	2.5		
Bwamba	4/10	2.89	3.5	0/8			++
Control	2/10	4.46		8/8	2.4		
Egypt	16/16	1.89	4.0	0/8			++++
Control	2/33	2.68		8/8	2.9		
Ilhéus	12/16	4.20	4.0	1/8	0.07	+	++
Control	2/33	2.68		8/8	2.4		
M.M.	4/12	3.7	4.0	4/4	0.7	+	+
Control	0/12	2.6		4/4	2.81		
Neurovaccinia .	All	1.47	2.0	2/8	0.4	+	—
Control	killed	0.85		8/8	1.22		
Ntaya	5/10	3.85	<1.0	5/8	1.56	+	—
Control	2/10	3.26		8/8	2.89		
Russian E.	15/20	0.8	2.75	1/8	0.04	<1.0	++
Control	12/20	1.21		3/8	3.4		
Semliki	10/10*	0.79	3.5	4/4	0.41	—	+++
Control	2/10	3.26		8/8	2.89		
Uganda	All	1.88	4.0	9/10	2.8	4.0	—
Control	killed	1.23		10/10	1.8		
Yellow fever ...	0/10	2.54	2.0	4/4	1.66	+	—
Control	2/10	3.26		8/8	2.89		
Zika	All	1.11	2.5	1/8	0.56	+	—
Control	killed	0.85		8/8	1.22		

* Some animals paralyzed.

Since these viruses have been tested for their destructive activity against the same tumor (HEP No. 3) growing in tissue culture, on the chorioallantoic membrane, and in the irradiated and cortisonized rat, it was possible to determine if there was correlation between the findings. Table 7 shows that if the virus is active in tissue culture there is good reason to think this will carry over into a solid tumor, the only exception being neurovaccinia which, although growing or surviving in the tumor, failed to influence its growth.

We end by pointing out that there still is something to this business of viruses destroying tumors—and human ones, too, but their possibilities as curative agents in patients is still nil. Recent experiments at the National Institutes of Health (Suskind *et al.*, 1957) in which the oncolytic ability of the Coxsackie virus was increased by passage in intraperitoneally implanted HeLa may provide a clinically hopeful lead.

TABLE 7

Effect of Virus on HEP No. 3 Grown in Eggs, Rats, and Tissue Culture

Virus	Tissue Culture	Egg	Rat
Anopheles A	++	—	—
Bunyamwera	++++	+++	++++
Bwamba	++++	+++	++
Egypt	++++	++	++++
Herpes	++++	++	ND*
Ilhéus	++	+	++
M.M.	++	++	+
Neurovaccinia	++++	ND*	—
Ntaya	†	—	—
Russian	++	+++	++
Semliki	++++	++++	+++
Uganda	++	+	—
Yellow fever	++	+	—
Zika	+	++	—

* Not done.
† Focal necrosis.

Discussion

Dr. Dmochowski: In your tissue culture experiments where you treated the cells grown *in vitro* by the various neurotropic viruses, have you observed during serial passages any increase in oncolytic properties, or decrease, as the case may be?

Dr. Moore: Actually, as measured by tissue culture methods, we have noticed both. It is quite difficult to determine if some of these viruses have increased in their oncolytic properties, since some of them—for example, Egypt 101—already destroy the HEP No. 3 tumor grown in the rat or on the chorioallantoic membrane of the chick. In this instance one must test it clinically to determine if it has increased in oncolytic ability. However, this same virus grown in the tumor HEP No. 2 only partially destroys it, and both cells and virus can be carried indefinitely so that in this case it does not increase its oncolytic ability. However, we did lose one of these cell lines, and I wondered if it were due to a change in the virus. I am sure with any given virus or cell system a change can occur in either direction.

Dr. Sabin: Dr. Moore mentioned some very disappointing aspects of the possible use of viruses as oncolytic agents in human beings. The most disappointing aspect is the fact that even when a virus is oncolytic, and it punches a hole in a tumor, the immune response of the individual to the multiplication of the virus occurs

so fast that the effects are quickly wiped out, and the tumor continues to grow. The question has been "what can you do about it?" Some people have thought in terms of trying to interfere with the immune response of the individual, but my own thinking is rather along different lines. I am thinking more along the military lines in this area. For example, if you have to take a certain position that is protected by an army of a great strength and you send in a few guerillas, they get wiped out. Military tacticians have handled problems of this sort by the system of successive waves of attack until a complete breakthrough is achieved. Now, what does it mean in terms of the application of viruses for the destruction of tumors? It means this—that you have to think about sending in not one virus or two viruses against the tumor and its metastases, but rather a whole army of viruses in rapid succession. Before one has been overcome by the immune effects of the individual, you send in another, and then another, and still another. Now then, where are you going to recruit this large army of viruses? That is part of the work that Dr. Moore has been talking about. She has tried to recruit her army from among those exotic agents that might not be found in the regions of the world where she is working. In other parts of the world these would be found and the people would be largely immune to begin with. I think that is very good, but in addition to that there are a great many more sources for recruitment. I have in mind particularly the adenoviruses and Echo viruses, not only of humans but also of chimpanzees and monkeys for which human beings have no antibodies. Furthermore, the number of Coxsackie viruses and Echo viruses in human beings is so great, that most people have antibodies for only a fraction of them. What is the best way to test them? I think it was encouraging that Dr. Moore found such a fine correlation between the cytopathogenic effects in tissue culture, and the effects on tumors grown in chick embryos or cortisonized rats. I think that the procedure she is following and the procedure that Dr. Huebner is now following, particularly to select more oncolytic variants from among the available viruses and not to rely only on naturally occurring strains, offers some promise. All in all, I think there is still a future for this line of work, and I think that with superior forces it may be possible to do something. Another point that comes to the mind of the military tactician, is that in inject-

ing these viruses, it might be a good idea to use the arterial supply of tumors and the arterial supply of metastases in order to bring most of the inoculated virus to bear at the point at which the attack is needed.

Dr. Montgomery: It occurs to me that you have done something very fundamental to the rats which was not done to the patients, and that was either treat them with total body x-ray, or with massive doses of cortisone during the time that they were being inoculated with the virus. I wonder if you have treated patients with massive doses of cortisone at the time that they were given the injection of the virus?

Dr. Moore: Yes, a small number of patients have been treated with cortisone. I think something like four, with adequate controls at the same time. But it didn't seem to make any difference. We have used cortisone, but no x-ray. We have used both cortisone and x-ray in an attempt to break down the immunity; that too was unsuccessful.

Dr. T. C. Hsu: I would like to request, Dr. Moore, if you would be kind enough to spend a minute and give us a resume of the inoculation of cell strains into patients, and the development of tumors. We have done quite a bit of transplantation studies, mostly from tissue culture material which has been inoculated into different types of individuals. These have been these tissue culture cells that you see here, and also the cells from normal sources which Dr. Chang has kindly given us. Briefly, we can say that these cell lines, both the tumor cell lines and the Chang cell lines do produce tumors or solid swellings in the cancer patients. The point I think that everybody wants to know is, is there any difference between the normal individual's reaction—a difference between the normal volunteers and the cancer patient volunteers?

Dr. Moore: There is. I can briefly summarize it, I think, just by saying that when we inoculate these cell lines into cancer volunteers, the usual course of events was that the swelling directly following the inoculation goes away. There might be a slight area of erythemia the next day. The inoculated site of the forearm is absolutely flat, and nothing happens for about a week. At that time, one can feel a small nodule which gradually increases in size. After ten to fourteen days, we remove the nodule com-

pletely by biopsy. That is the picture in the far-advanced cancer patient. In the normal volunteers we got an altogether different reaction. After the inoculation of the same cell types there was a large amount of the swelling, with edema, redness and induration which persisted for a matter of a week, and then it gradually subsided at two weeks. In line with what we did with our cancer volunteers, these swellings were removed, and in a small percentage of these individuals we did find cancer cells. But their appearance was altogether different in that there was much reaction, and the cells did not look at all happy. This was in contrast to the cancer patients.

Dr. Dudley Jackson, Sr.: Back in 1938 I was to read a paper before the International Cancer Conference reporting on transplanting some cancer into human beings. Dr. F. C. Wood said at that time that the anti-vivisectionists would be upset and refused to let me read the paper. But after I had transplanted into myself some basal cells of cancer, with Dr. McCoy's help I was permitted to present the report. I think that this beginning to be a rather pointed thing, because I do not have cancer. Nineteen years have passed, and God has been good to me up to this date.

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FILTERABLE AGENT CAUSING LEUKEMIA FOLLOWING INOCULATION INTO NEWBORN MICE*

LUDWIK GROSS

Attempts to transmit mouse leukemia by filtered extracts succeeded, when newborn mice of a susceptible strain were used for inoculation (Gross, 1951).

Some of the leukemic extracts were very active, inducing leukemia or other types of tumors in a considerable number of injected mice after a relatively short latency; other extracts were less potent, inducing leukemia or tumors only occasionally, and after a long latency. Among the extracts used for inoculations, some, though prepared from typical leukemias and under apparently identical experimental conditions, revealed no pathogenic potency on inoculation assays (Gross, 1956; Woolley, 1956).

The fundamental feature of these studies was the use of newborn, less than 16 hours old, suckling mice for the inoculation of the cell-free centrifuged or filtered, leukemic extracts (Gross, 1951–1953). When older suckling mice were used for inoculation, the results were erratic, and the development of leukemia considerably delayed, if it occurred at all. Normal adult mice were with few exceptions resistant to inoculation of the cell-free extracts.

Another fundamental feature of these studies was the strain specificity determining the susceptibility of the hosts to inoculation of the cell-free extracts. Some strains were resistant. Newborn C3H mice of the Bittner substrain were found to be substantially more susceptible to the leukemogenic action of the cell-free extracts, than mice of the National Cancer Institute substrain of the same inbred line (Gross, 1955). On the other hand, certain lines, such as C57 Brown/cd, were found sensitive to the leukemogenic action of the extracts only (Gross, 1954), whereas mice of other strains, such as C3H, reacted in a different manner.

A striking and unexpected result was the development, among

* From the Cancer Research Unit, Veterans Administration Hospital, Bronx 68, New York, and the Sloan-Kettering Institute for Cancer Research, Memorial Center, New York. Aided, in part, by grants from the Damon Runyon Memorial Fund, and from the American Cancer Society.

some of the inoculated C3H mice, of tumors different from leukemia, and rarely, if ever, occurring spontaneously in untreated mice of the strain used for inoculation. The tumors induced with the cell-free leukemic extracts were either parotid gland carcinomas (Gross, 1953), or subcutaneous fibro-myxo-sarcomas, occasionally medullary adrenal tumors, or mammary carcinomas (Gross, 1955). When, in turn, cell-free extracts were then prepared from the induced leukemias, parotid tumors, or sarcomas, and then inoculated into newborn C3H mice, their pathogenic potential was highest when prepared from induced leukemias (50 per cent), substantially less so when prepared from parotid tumors (33 per cent), and rather weak when made from the subcutaneous sarcomas (16 per cent). Regardless, however, whether prepared from the induced C3H leukemia, parotid tumors, or subcutaneous sarcomas, such cell-free extracts, when inoculated into newborn C3H mice, were able to induce, though in different degree, either leukemia (7 per cent to 37 per cent), parotid tumors (5 per cent to 10 per cent) and/or fibromyxosarcomas (4 per cent to 10 per cent) (Gross, 1956).

TABLE 1

Results of Inoculation of Filtered Ak Leukemic Extracts Into Newborn C3H or C3H(f) Mice

FRESH FILTRATE				HEATED FILTRATE (65° C. to 68° C. ½ hr.)				
No. of Mice Inoc.	No. Dev. Leuk.	No. Dev. Parotid Tumors	No. Dev. Subcut. Sarcoma	No. of Mice Inoc.	No. Dev. Leuk.	No. Dev. Parotid Tumors	No. Dev. Subcut. Sarcoma	No. Died Free From Leuk. or Tumors
328	92 (28%)	36 (11%)	3 (1%)	166	2 (1%)	5 (3%)	0	159

Of 70 extracts prepared, 18 were inactive.
Average age, months, developed leukemia: 10, parotid tumors: 5, sarcoma: 13.
Average age died free from leukemia or tumors 16 months.

The development of leukemia in some of the injected mice, and that of parotid tumors and/or subcutaneous fibro-myxosarcomas in others, was quite unexpected, and raised immediately fundamental questions, which have not yet been fully answered.

The basic question remains to be answered whether one agent inoculated into newborn C3H mice is able to induce either leu-

kemia, parotid carcinomas or subcutaneous sarcomas, or whether 3 or more distinct oncogenic agents are present in such extracts.

It is of considerable interest, that mouse leukemia transferred from one mouse to another by the conventional method of cell-graft would reproduce leukemia only in the inoculated hosts. When, however, cell-free, centrifuged or filtered, extracts were prepared from leukemic organs, and inoculated into newborn mice of a susceptible strain, the result was not always leukemia, but in some instances carcinomas (of the parotid glands) or fibro-myxo-sarcomas. Again, when such carcinomas, or sarcomas,

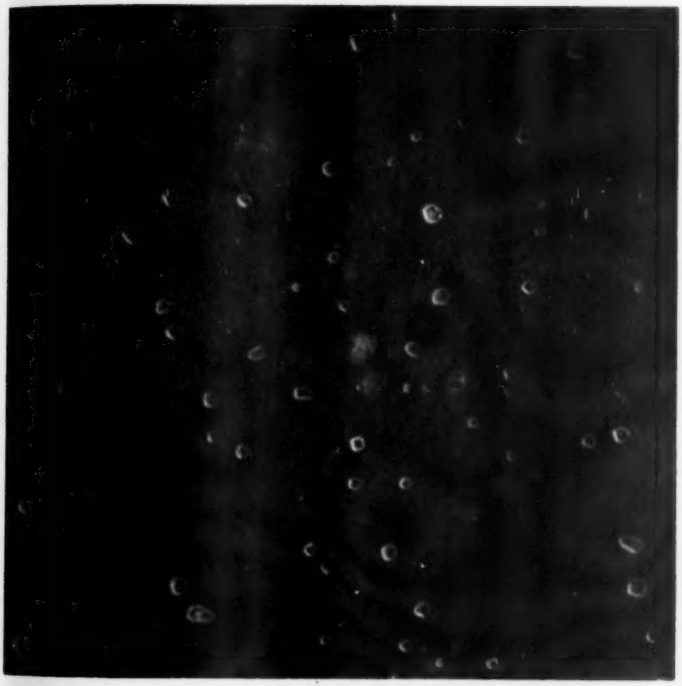
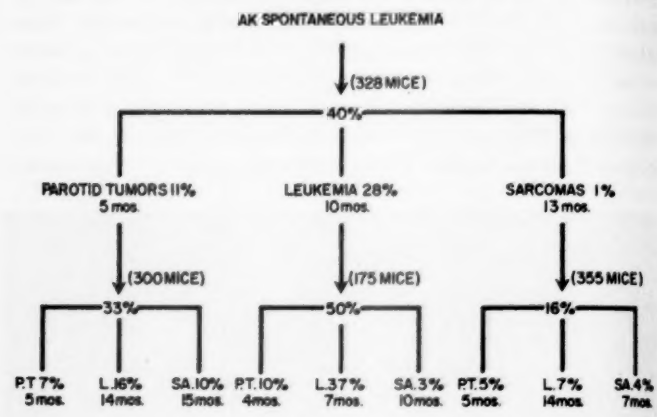


FIG. I. Electronmicrograph of a leukemic extract prepared from a C3H mouse in which leukemia was induced with passage-leukemia agent. This extract, prepared from spleen, liver, and lymphoid tumors, was, after centrifugation, filtered through a Selas, porosity 02, filter candle. Numerous particles are seen, most of them spherical, varying in diameters from 30–78 mu, average size 57 mu. Note doughnut-like structure. (Drop preparation, chromium shadowed, RCA electron microscope EMU-2B). x 33,000.

FIGURE II.

RESULTS OF INOCULATION OF CELL-FREE (CENTRIFUGED OR FILTERED) EXTRACTS INTO NEWBORN C_3H MICE



were transplanted to other hosts by the conventional method of cell-graft, identical tumors were reproduced, *i.e.*, carcinomas or sarcomas, respectively. When, however, cell-free extracts were prepared from either carcinomas or sarcomas, and inoculated into newborn, susceptible mice, in some instances at least, leukemia resulted.

Obviously, one could explain this by assuming that the same agent might have caused either disease. It is also possible to speculate, however, that a mixture of distinct oncogenic viruses was present in the leukemic extract. Mouse leukemia, or mouse carcinoma or sarcoma, may carry different oncogenic agents, even though one of them only at a time may assert itself and induce a corresponding tumor. The conventional transfer of a tumor by graft may not reveal the presence of all potential oncogenic agents; a filtrate, however, prepared from a tumor, and injected into a susceptible host under proper experimental conditions, may reveal the presence of hitherto obscure, though present, oncogenic agents. This would explain the results obtained by Graffi and his co-workers (1950). In their experiments filtered extracts prepared from a transplanted carcinoma or sar-

coma, and inoculated into newborn mice, induced frequently typical leukemia.

Similarly, in recent studies, Carr (1956) induced renal adenocarcinomas in young chicks inoculated with leukemic filtrate. In these experiments very young hosts had to be inoculated with the cell-free extracts in order to induce renal carcinomas. This was

TABLE 2

Results of Inoculation of Centrifuged or Filtered Extracts Prepared From Ak-Leukemia-Induced C3H Leukemia, Into Newborn C3H Mice

No. of Mice Inoc.	No. Dev. Leuk.	No. Dev. Parotid Tumors	No. Dev. Subcut. Sarcoma	No. Died Free From Leuk. or Tumors
175	64 (37%)	17 (10%)	5 (3%)	89

Of 25 extracts, 6 were inactive. 2 mice developed parotid tumors and leukemia. 4 mice developed parotid tumors and sarcoma.
Average age, months, developed leukemia 7, parotid tumors 4, sarcoma 10. Average age died free from leukemia or tumors, 14 months.

TABLE 3

Results of Inoculation of Centrifuged or Filtered Extracts Prepared From (Leukemia-Induced) Parotid Tumors Into Newborn C3H Mice

No. of Mice Inoc.	No. Dev. Leuk.	No. Dev. Parotid Tumors	No. Dev. Subcut. Sarcoma	No. Died Free From Leuk. or Tumors
300	47 (16%)	20 (7%)	29 (10%)	204

Of 38 extracts, 10 were inactive. 6 mice developed parotid tumors and subcutaneous sarcoma.
Average age, months, developed leukemia 14, parotid tumors 5, sarcoma 15. Average age died free from leukemia or tumors, 16 months.

TABLE 4

Results of Inoculation of Centrifuged or Filtered Extracts Prepared From Induced Sarcomas Into Newborn C3H Mice

No. of Mice Inoc.	No. Dev. Leuk.	No. Dev. Parotid Tumors	No. Dev. Subcut. Sarcoma	No. Died Free From Leuk. or Tumors	No. Alive and Free From Leuk. or Tumors
355	26 (7%)	19 (5%)	13 (4%)	260	37

Of 35 extracts, 18 were inactive.
Average age, months, developed leukemia 14, parotid tumors 5, sarcoma 7.
Average age died free from leukemia or tumors, 16 months.
Average age alive free from leukemia or tumors, 11 months.

also true in our experiments in which filtered mouse leukemia extracts induced parotid tumors when inoculated into newborn C3H mice, but did not prove pathogenic when injected into adult animals.

Should results of further studies be consistent with the assumption that the leukemic hosts carry different oncogenic agents, it would not be surprising to find such agents to be related, though possibly of a different pathogenic potential.

Recent Development and Serial Passage of a Potent Mouse Leukemia Virus

Since extracts prepared from C3H mice in which leukemia had been induced with cell-free Ak leukemic agent proved to be more active than those prepared from Ak mice with spontaneous leukemia, it appeared possible to assume that a single cell-free passage of the leukemic agent from one host to another may already increase the leukemic agent's pathogenic potential.

An attempt was therefore made to select a particularly active extract among the many tested in our laboratory, and then carry such an extract through successive cell-free passages, inoculating in each passage groups of newborn hosts. Among the inoculated mice, those developing leukemia at earliest date were then used as donors for the preparation of new cell-free extracts, which were then again inoculated into newborn mice.

This experimental procedure was repeated several times. At least two different highly potent passage strains of leukemic agents were thus obtained, one originating from a C58 (Gross, 1956), the other from an Ak donor, with spontaneous leukemia (Gross, 1957).

One of these two passage strains, originating from Ak spontaneous leukemia, and designated "passage A" proved to be of particular interest. After six consecutive cell-free passages through newborn hosts, this passage strain of mouse leukemia proved to be highly pathogenic. Filtered extracts prepared from the 6th, 7th and 8th passages of this agent induced, when inoculated into newborn C3H mice (Bittner substrain), acute generalized lymphatic leukemia in over 90 per cent of the inoculated mice after a latency period usually not exceeding 3½ to 4 months. Furthermore, extracts containing the leukemic passage

virus could be preserved by lyophilization, or simply by freezing in carbon dioxide ice at —70° C. in sealed ampoules for periods of time up to 5 months, without apparent loss of activity (Gross, 1957).

It is hoped that successive passages will continue to induce leukemia in most of the inoculated mice, and that, perhaps, the latency period may also become shortened. Should this trend continue, it may then become possible to duplicate in experiments on mouse leukemia the results thus far obtained with the virus of chicken sarcoma (Bryan *et al.*, 1955), and chicken myeloblastosis (Beard, 1956).

The results thus far obtained with the mouse leukemia passage

TABLE 5

Results of Inoculation of Centrifuged or Filtered Extracts Prepared From Leukemic Passage a Strain Into Newborn C3H Mice

Pass. No.	No. of Extracts Inoc.	No. of Liters Inoc.	No. of Mice Inoc.	No. Dev. Leuk.	Average Age Months	Leuk. Inc. Per Cent
1*	1	1	7	6	6	86
2	1	1	3	3	3	100
3	1	1	5	2	7	40
4	1	4	7	3†	6	43†
5	1	2	5	5	5	100
6	3	5	12	11	7	92
7	4	10	37	37	3	100
8	2	7	20	19	3	95

* First extract prepared from 7½ months old Ak female with spontaneous leukemia.
† 2 additional mice developed parotid tumors. Total incl. of leukemia and tumors, 86%.

TABLE 6

Results of Inoculation of Centrifuged or Filtered Extracts Prepared From Leukemic Passage 6 and 7 A Into Newborn C3H Mice

Pass. No.	No. of Extracts Prep.	No. of Liters Inoc.	Extract Inoc.	Route of Inoc.	No. of Mice Inoc.	No. Dev. Leuk.	Average Age Months
				s.c.	5	5	4
			Ctr	i.p.	4	3	4
6	3	5					
			Fil	i.c.	3	3	3
			Ctr	s.c.	9	9	2.5
7	4	13					
				s.c.	22	22	3
			Fil	i.c.	4	4	3
				i.p.	7	7	3.5

S.c., subcut.; i.p., intraperit.; i.c., intracranial.

agent could be compared to studies with the Rous sarcoma virus recently reported by Bryan and his associates. At first, when the filterable chicken tumor was discovered by Rous (1911), it was not always possible to duplicate cell-free transmission using any tumor-carrying-chicken as a donor for the preparation of the filtrate. Gradually, it has been learned that different tumors contain different amounts of infective virus; cell-free passage of the Rous sarcoma virus from one host to another gradually increased the potency of the obtainable extracts. It is now possible to induce sarcomas in chicks with cell-free extracts in 100 per cent of cases after a short latency of only 1 to 2 weeks. Ampoules containing the chicken tumor agent can be kept at —70° C. and used as needed for inoculation of 3-week-old chicks of a susceptible strain. In the work with chicken sarcoma and chicken myeloblastosis, the selection of a susceptible strain of hosts for inoculation of the virus is as important as in the work dealing with experimental transmission of mouse leukemia. It is equally important, however, to use potent extracts for inoculation (Bryan *et al.*, 1955).

A potent filtered extract inducing leukemia in most of the inoculated mice within a relatively short period of 2 to 3 months, would make it possible to study many biological, physical, and pathogenic properties of the mouse leukemia virus and its possible relation to the agent or agents causing parotid gland tumors and subcutaneous fibro-myxo-sarcomas. Such highly potent extracts, which also could be preserved at —70° C., would be welcomed in many laboratories, to replace the unpredictable pathogenic potential of leukemic extracts prepared from different Ak or C58 donors with spontaneous leukemia.

It is now apparent that the initial difficulties encountered in attempts to duplicate our first published reports on cell-free transmission of mouse leukemia were due not only to the low susceptibility of recipient mice (Law *et al.*, 1955), but probably also to the low infective potential of the extracts used for inoculation.

Only Newborn Mice Susceptible to Inoculation of the Cell-Free Agent

It should also be emphasized that the cell-free, centrifuged or filtered extracts, prepared from the highly potent passage strain

A of mouse leukemia reported in this paper proved to be consistently pathogenic only when inoculated into newborn C3H mice. These mice were in most instances less than 12, never more than 16, hours old at the time of inoculation. In each experiment, young adult (1½ to 3 months old) C3H mice have been also routinely inoculated. Thirty-five filtered extracts were prepared, each from a different leukemic donor, and inoculated into a total of 116 young adult C3H mice. Only 4 out of 94 adult C3H mice inoculated with the leukemic filtrates intraperitoneally (0.5 to 1 ml. each), and 1 out of 22 inoculated intracranially (0.1 to 0.2 ml. each), developed leukemia. These were apparently exceptions, which could not be duplicated at will.

Thus, in all these experiments, including those in which the highly potent passage-strain of the mouse leukemia virus was used, newborn mice, less than 16 hours old, had to be injected in order to induce leukemia. In this respect, therefore, the mouse leukemia agent used in our studies appears to be fundamentally different from that recently reported from the Sloan-Kettering Institute in New York. When Friend inoculated cell-free extracts prepared from a transplanted Ehrlich ascites mouse carcinoma into newborn Swiss mice, some of the inoculated mice developed generalized leukemia 14 months later; one of these leukemias, a myleogenous form, proved to be transmissible by filtered extracts into adult Swiss mice (Friend, 1957); in this particular case, newborn hosts were not required for the successful transmission of the cell-free agent. It is conceivable, therefore, that there may exist different mouse leukemia viruses, some transmissible to newborn hosts only, others to adult hosts as well. Beard and his associates made similar observations in their studies on chicken leukemia: whereas chicks less than 3 days old had to be used for inoculation with the myeloblastosis virus, young adult chickens could be inoculated with the virus of erythroblastosis (Beard, 1956; Beaudreau *et al.*, 1956; Eckert *et al.*, 1955).

Tumor Inducing Carcinogens and Oncogenic Viruses

The apparently disturbing fact is that leukemia can be induced in most laboratory strains of mice by total body x-ray irradiation, and in some strains at least, by estrogenic hormones or with certain carcinogenic chemicals, such as methylcholan-

threne. Theoretically, therefore, one might be inclined to classify the transmissible leukemic agent described in our study as just another leukemia-inciting factor, comparable to ionizing radiation, hormones, or carcinogens. Those who would favor such an interpretation, however, would have to reconsider the generally accepted assumption that such tumors as Rous sarcoma, or mouse mammary carcinoma, are caused by specific filterable viruses. Although either of these tumors can be induced in a susceptible host by inoculation of a specific filterable virus, sarcomas in chickens can also be induced with chemical carcinogens, and mammary tumors in mice can be induced with either methylcholanthrene or with estrogenic hormones.

The fact that ionizing radiation, hormones, or carcinogenic chemicals may be able to induce tumors and/or leukemia, does not necessarily imply that these tumor-inducing factors are similar in their pathogenic action to oncogenic viruses. Ionizing radiation, hormones, and carcinogenic chemicals are able to exert their oncogenic potential on different species of hosts, and as a rule they cannot be recovered from tumors which they have induced. The tumor-inducing viruses, however, are limited in their pathogenic potency to single species, and even to particular inbred lines within a species; furthermore, in many, if not most, instances oncogenic viruses can be recovered, frequently even with increased potential, from tumors which they induced. This is not true for chemical carcinogens, or hormones, which are metabolized, decomposed, and/or excreted.

It is entirely possible, though purely speculative at this time, that ionizing radiation, hormones, or chemical carcinogens merely activate latent oncogenic viruses; their tumor-inducing action could thus be compared to the experimental induction of bacteriophage in lysogenic bacteria. Should this concept prove to be true, oncogenic viruses could be compared to temperate bacteriophages, transmitted from one generation to another in lysogenic bacterial hosts, only very occasionally killing their carriers (Gross, 1954).

In any event, it was demonstrated that mouse leukemia could be induced with a thermolabile, filterable agent; in order to induce disease, this agent had to be injected into newborn mice, of a susceptible strain; this agent could then be recovered from the induced disease, and serially transmitted, from one host to an-

other; it is apparent, therefore, that mouse leukemia is caused by a specific, filterable virus.

In further experiments it was demonstrated that carrier mice of leukemic strains, earmarked to develop leukemia at middle age, even though in apparent good health when young, transmit the virus to their offspring. This transmission occurs not through the milk, as in the case of mammary carcinoma, but directly through the embryos, from one generation to another (Gross, 1951). Mouse leukemia, as chicken lymphomatosis (Cottral *et al.*, 1954), appears to be, therefore, an egg-borne virus disease (Gross, 1954–1956). As in the case of chicken lymphomatosis, however, in mouse leukemia also, other routes of transmission of the virus would have to be considered.

Formal Discussion

Dr. Woolley:* During the past two years we have been investigating the relation of cell-free extracts prepared from leukemic tissues—centrifugates and filtrates—to the induction of cancer, particularly leukemia, in mice.

These studies were started because of the interesting observations made by Gross that cell-free virus-like material prepared from either leukemic tissue or normal tissue from leukemic-strain mice would induce leukemia and other forms of cancer when given to new-born mice of different susceptible strains.

Materials and Methods

The strains of mice used in this study were AKn/Gs (Gross), C3H/Gs,C3H$_f$/Gs (f=fostered to eliminate the virus-like milk factor), C3H$_f$/Bi (Bittner), C3H$_f$/Hu (Hummel), and C3H/Wy (Woolley).

The extracts were inoculated into newborn mice less than 12 hours old in most instances, frequently less than 6, and never more than 16. After weaning, the mice were carefully examined at weekly intervals for tumor formation.

The control mice were obtained from alternate litters of the same parental mice as the treated individuals and were maintained in small groups of five or six as nonbreeders.

The methods of preparation of the extracts and injection into newborn mice have followed closely those developed by Gross.

* See References.

Twenty per cent pooled preparations of ground thymus, liver, spleen, and lymphoid tumors were prepared with chilled (4° C.) physiological saline, centrifuged at 3000 r.p.m. (1800xg) for fifteen minutes and then at 9500 r.p.m. (7000xg) for ten minutes at 0° C. The final supernatant (centrifugate) was used for inoculation or passed through a Selas 02 or 03 porosity filter under vacuum pressure of approximately 20 to 25 mm. of mercury (filtrate) prior to use. The centrifuged extract (10 cc.) was mixed with a diluted broth culture of *Escherichia coli* (0.5 cc.) prior to filtration. The filtrates were bacteriologically sterile as evidenced by inoculation of ordinary culture media, so it may thus be assumed that these filtrates were cell free. All extracts were kept close to 0° C. in ice water and injected (0.05 cc.) subcutaneously within forty-eight hours after preparation.

Portions of the tumors that developed were fixed in Vandergrifft's fluid, sectioned, and studied histologically. Liver sections were secured for aid in leukemia determination. Many of these tumors were transferred by trocar or cell suspension to mice of both the strain of extract and the strain of tumor origin for analysis of genetic status.

Results

The development of leukemia and/or other types of tumors appeared to depend to a large extent upon the preparation used and the genetics of the host receiving the preparation.

At the present time the results are nearly completed for the first groups. Of thirty-one extracts (twenty of/AKn/Gs origin and eleven of $C3H_f$/Gs origin), a total of sixteen (eight AKn and eight C3H) have been active in inducing leukemias, parotid gland tumors, and sarcomas. The most active preparations were extracted from filtrate-induced C3H tumors rather than from spontaneous AKn tumors. One of the C3H filtrates has induced five leukemias out of five inoculated $C3H_f$/Bi mice (100 per cent) and eleven in seventeen $C3H_f$/Gs mice (65 per cent).

Use of a supernatant that was diluted with an amount estimated to be 2:1 (two parts of supernatant to one part of saline), after 7000xg centrifugation, was followed only by parotid-gland tumors and sarcomas-seven and seven respectively in seventeen injected $C3H_f$/Gs and one sarcoma in three injected C3H/Gs mice.

Use of one AKn centrifugate was followed by seven leukemias out of twelve injected mice at an average age of 8.5 months. Another AKn preparation was refrigerated at 4° C. in an ice bath for an excessive period of time, namely seventy-nine hours, before use. Of the four $C3H_f$/Bi mice injected with this extract, four parotid-gland tumors and three sarcomas resulted. One leukemia and two sarcomas have occurred in the six $C3H_f$/Gs mice that were injected with this extract within forty-eight hours after preparation. These parotid-gland tumors and sarcomas were the only ones in the early AKn extract series.

All types of tumors were transplantable. Tumors arising in C3H mice were transplantable to other C3H mice of the proper subline but not to AKn mice even though the filtrates were of AKn origin.

Discussion

The experiments reported in part in this paper have confirmed the fact that mouse leukemia can be transmitted under certain experimental conditions by inoculation of cell-free (Selas-filtered) leukemic tissue extracts. The extracts apparently must be inoculated into newborn mice and into a susceptible strain.

The question remains to be answered as to why, among the inoculated animals, some eventually develop leukemia, while others develop parotid-gland tumors and/or subcutaneous sarcomas. The inoculated extracts may well contain a single oncogenic agent potentially able to induce different types of neoplasms. On the other hand the possibility must be considered that the extracts used for inoculation contain a mixture of several distinct agents. These possibilities have recently been discussed by the author in more detail.*

Summary

The relation to leukemia and to other cancers of cell free extracts—centrifugates and filtrates—prepared from leukemic mouse tissues is being studied. At the present stage of development, it is concluded that these studies confirm the new and basic findings by Gross that mouse leukemia can be transmitted by cell-free extracts (Selas filtered) when inoculated into newborn

* George W. Woolley and Marcia C. Small: Experiments on Cell-Free Transmission of Mouse Leukemia. *Cancer*, 9: 1102– 1106, Nov.–Dec. 1956.

mice of a susceptible strain. Under certain, not fully determined, conditions the extracts are able to produce parotid-gland tumors and/or subcutaneous sarcomas.

When extract transfer was made from strain AKn to C3H the induced tumors were of C3H type rather than AKn type as determined by tumor transplantation.

Formal Discussion

Dr. Ida:* We set up our experiments in 1954 along the same lines as Dr. Gross's experiments. At that time newborn C3H mice were injected with Seitz-filtered extracts of leukemic tissue obtained from spontaneous C58 leukemias. Five leukemias appeared in 61 inoculated male mice, an incidence of 8 per cent. This does not, however, represent an increase over the spontaneous incidence in this strain—22 leukemias occurring in 164 untreated C3H male mice. If questionable cases are included, and these in all probability are leukemias, the incidence is 19 per cent (31 cases in 164 mice). Out of this group of 164 male mice, it is to be emphasized that only one animal developed leukemia before 13 months of age. Another point of emphasis is the need for careful observation of old mice which are kept in good condition. Reports from other laboratories are confirming our impression that the C3H strain is low-leukemia only in the sense that leukemia does not appear during the first year of life. If females are studied, the incidence of leukemia is very low since the mice die from mammary cancer before reaching the leukemia age.

Since the leukemogenic agent described by Dr. Gross may have been absorbed onto the asbestos of the Seitz filter, unfiltered extracts were inoculated into newborn C3H mice. These extracts were prepared in the cold according to the method of Dr. Gross, and were centrifuged 20 minutes at 3,000 r.p.m. followed by centrifugation at 7,000 gravity 10 minutes. The supernatant was inoculated. In the C3H male mice inoculated as newborns with the unfiltered extracts, 5 leukemias have developed before 13 months of age with 14 mice dead; of the 45 living mice all are less than 14 months of age. It can be anticipated that more cases of leukemia will occur in this group. In a group of 56 females similarly inoculated, 2 leukemias have appeared early.

Our interpretation of these results is the following: Leukemia appears spontaneously in C3H mice, but only after 13–14 months

of age, when the incidence is probably approximately 20 per cent. The disease appears earlier if C3H mice are inoculated at birth with cell-free extracts of leukemic tissue derived from spontaneous cases of the C58 strain of mice. This is in agreement with the findings of others who have observed an acceleration of onset of leukemia in high leukemia strains inoculated at birth with leukemic tissue extracts.

When C57 BL mice were inoculated at birth with *filtrates* obtained from C58 tissue extracts, leukemia was not induced. The incidence in control males is 1.9 per cent, females 8.1 per cent. It is apparent from the data shown that the incidence in inoculated mice will be no higher than in controls.

When C57BL·C3H F_1 hybrids were used as recipients for filtrates no significant difference was found between the filtrate-inoculated and untreated groups. In these F_1 hybrids it is to be noted again that although leukemia does occur spontaneously, in none of this entire group of 220 mice did the disease appear before 17 months of age. The F_1 hybrids have a greater life expectancy than the pure parent stocks, and the disease appears even later in life.

In one series of experiments viable leukemic cells were inoculated into newborn mice. The newborn recipients were C3H mice and the donor leukemias were either C58 (spontaneous) or DBA (carcinogen-induced).

In general, the leukemias developing in the recipients can be considered early or late. The early leukemias, occurring within 3 months after transplantation, developed directly from the inoculated C58 cells which apparently survived, proliferated and infiltrated the recipient's organs. When these leukemias were transplanted they grew uniformly in C58 but not C3H mice. Of 4 leukemias which appeared 4 or more months after inoculation of C58 cells, one has transplanted so far to C3H, but not C58 mice, suggesting that this is a leukemia originating from C3H tissue rather than inoculated C58 leukemic cells.

Similarly, 5 leukemias have appeared late in C3H mice inoculated as newborns with the DBA leukemic cells derived from carcinogen-induced leukemias. Thus far one of these has grown upon transplantation into C3H, but not DBA mice.

If, in these experiments, the leukemias are genetically C3H either one of two phenomena has occurred. Either the inoculated

cells have adapted to the recipient, growing now as though the host were their source, or the leukemic cells have actually arisen from the recipient, the cellular inoculum providing the leukemogenic force.

Formal Discussion

Dr. Kirschbaum:* There are many questions that are raised by the experiments of Dr. Gross. I would like to bring some of these up at this time, and I hope that they will be discussed.

In the first place, what do we mean by a "low leukemia" strain? During the past twenty years we have studied fifteen inbred strains of mice, and leukemia occurs in every one of them. The incidence is low, and the disease appears late in the so-called "low leukemia" strains. Is there no filterable agent; is the disease developing independently insofar as an agent is concerned? Is there a small amount of agent, or are these animals genetically resistant to the action of an agent? If these mice which develop leukemia do have an agent, why is it that the incidence of leukemia is no higher in their progeny than in the remaining mice of the strain?

Now, concerning this question as to whether or not the disease can appear without the action of an agent. There may be multiple factors involved in the genesis of mouse leukemia, as in the genesis of mouse mammary cancer, and depending on the potency of certain of these factors, others may be deleted. I would like to make reference to the work on mouse mammary cancer which is now classical. When Bittner first fostered C3H mice on females of low mammary cancer strains, the incidence was less than 1 per cent. From the mammary cancers which developed, no agent could be identified; no agent could be revealed using the same method that had so readily demonstrated the agent in the cancers appearing in the animals of the high mammary cancer sublines. Heston has found, however, that when C3H mice are fostered in his laboratory, the incidence of mammary cancer may be as high as 38 per cent, and from these mammary cancers no agent can be revealed. Muhlbock has made pituitary isografts into these fostered C3H mice, and the incidence of mammary cancer has been nearly 100 per cent. And from these mammary cancers no agent has been identified, using the method which is routinely used for its demonstration. Heston and Muhlbock feel that the agent is an accelerant, and that the disease can appear in

its absence. Now, this is something which can be debated. Is this difference quantitative or qualitative? In other words, is the agent absent, or is the amount of agent very small? Is it present in a state making its demonstration difficult? Here, in the experiments of Dr. Ida, we have the suggestion that the extracts given at birth accelerated the onset of leukemia. I would like to emphasize the fact that we must study older non-inoculated mice very carefully, just as carefully as we study inoculated animals, and I am sure that leukemia will be found late in life in other sublines of the C3H strain. As to whether or not this is leukemia that appears late—it most certainly is—these leukemias are transplantable.

We have frequently raised the question as to whether or not x-ray-induced or carcinogen-induced leukemia has anything to do with a filterable agent. After all, in the experiments of Dr. Gross it is necessary to introduce the agent early in life. Presumably the agent is not present in active form unless it is so introduced. However, one can take mice of specific genetic strains that are 6 to 10 weeks of age, irradiate them with x-rays, or treat with carcinogenic hydrocarbons, or administer estrogenic hormone, and leukemia does appear. I am glad to hear that Dr. Gross has assayed these induced leukemias for agent; apparently he has revealed none—at least, with his present assay method. This raises the question as to whether or not leukemia might not be a common response to multiple agents including a virus. We all know of the classical experiments on the induction of skin cancer in rabbits. One can obtain skin cancer which is virus-induced by the Shope papilloma virus, and one can also induce skin cancer with tar. In the first case, a filterable agent is revealed, in the second instance it is not; the agent is apparent only in the precancerous or early Shope lesions (papilloma), but not when the carcinoma appears. Although the spontaneous control incidence of leukemia in Dr. Gross's subline is two out of 166 mice, these two cases still have to be explained. Why do they appear? Why do they appear if the C3H strain has no agent? All of the mouse neoplasms (virus-associated) in question such as parotid tumors, fibrosarcomas, and leukemia may appear spontaneously in low incidence in specific strains of mice. Heston has recently reported that late in life C3H mice develop fibrosarcomas of the skin. Law has observed spontaneous parotid tumors in C3H mice.

Dr. Ida has observed late "spontaneous leukemia" in the same strain. Is a filterable agent essential for the development of each of these neoplasms, or does a filterable agent accelerate onset?

Discussion

Dr. Gross: I should like to mention that among the mice inoculated, when newborn, with the filtered extracts, we have seen some which developed not necessarily leukemia, parotid tumors, or sarcomas, but certain other tumors such as medullary adrenal tumors, or mammary carcinomas. We have seen however, very few medullary adrenal tumors, not more than perhaps 2 per cent among our inoculated mice. The mammary carcinomas which developed in some of the inoculated mice as early as at the age of 2½ or 3 months, were puzzling.

Our attempts to recover a filterable agent from x-ray-induced leukemia, will be discussed in a more detailed manner at the forthcoming 48th Annual Meeting of the American Association for Cancer Research in Chicago. At this time I can state that only occasionally could we induce leukemia or parotid tumors with filtered extracts prepared from donors with x-ray-induced leukemia.

Concerning the incidence of spontaneous leukemia in untreated C3H mice, it is of course unfortunate that we do not have a completely leukemia-free indicator host; in other words, we do not have strains of mice which would be susceptible to the leukemogenic action of the cell-free leukemic extracts and which would be at the same time completely free from spontaneous leukemia. A similar situation exists, however, in work with many other tumors, including mouse mammary carcinoma, chicken lymphomatosis and Rous sarcoma.

Dr. De Carvalho, Cleveland, Ohio: Has any attempt been made to learn where in the leukemic cell Dr. Gross's agent is located, or did he fractionate leukemic cells and use some of the fractions in the way he uses his filtrates?

Dr. Sabin: I have followed the work of Dr. Gross with great interest for many years. One of the greatest difficulties in this work has been that you had to wait for two years in order to have an experiment completed. So this work has represented as frustrating and difficult an endeavor in which anyone could engage. For many years there was none who attempted to confirm what Dr. Gross had done. Now today, we are confronted with two dif-

ferent sets of data for discussion. Dr. Woolley presented data which he carried out after having obtained directions from Dr. Gross and with control animals observed at the same time. The data presented complete confirmation of Dr. Gross's observations. On the other hand the Houston workers, using techniques which are somewhat similar, presented the evidence in which one could find no significant difference between the rate of leukemia occurring in control animals and those inoculated with leukemic extracts. The rate in their control animals appears to be much higher during the same period of time than that observed by Dr. Gross and Dr. Woolley. I hope that both Dr. Woolley and Dr. Gross will comment on this difference about the activity of leukemic extracts in so-called low leukemic strains of mice. I don't think that Dr. Gross had enough time to describe how he suddenly got the very active extract which now produces leukemia in 100 per cent of mice in about 3 to 4 months. He said he used Bryan's technique, but I am not sufficiently acquainted with that. It also appears that he might have used some method of selection, because he always made an extract from the first mice to develop leukemia. Could he perhaps say a little bit more about that? The most significant point in the phenomenon described by Dr. Gross is the importance of inoculating the mice shortly after birth. He was undoubtedly influenced by the discovery of the coxsacki viruses by this procedure, but is viral activity the only possible explanation for the role of the newborn mouse in this phenomenon? Perhaps we should also consider here the phenomenon of transformation of pneumococci and transduction generally where the "transforming" DNA has to be added to the bacteria during a certain phase in its metabolism or growth cycle in order to be absorbed, and to exert its effect. And so, I think, that in cell-free extracts of tumors exhibiting a virus-like action it is very important to determine whether or not the phenomenon of transduction may be operative—that is to say whether the nucleic acid is the culprit. If it is a virus, we would not expect the exzymes capable of destroying DNA or RNA to have any effect. If, on the other hand, the active factor is DNA or RNA one would expect destruction by the corresponding enzyme. I know that Dr. Gross has already undertaken experiments to check this possibility. Until the results of such tests become available, we might perhaps postpone a decision as to

whether the active principle in the leukemic is a virus or nucleic acid.

Dr. Syverton: Have you attempted to employ graded temperatures to 65° for separation of what appear to be three or four etiologic agents? Deficient viruses may be separable by comparative heat resistance.

Dr. Kirschbaum: I don't think the discrepancy is as great as Dr. Sabin suggested. If you will recall, leukemias did appear early, or are appearing earlier in the animals which were injected with the *unfiltered* extract. Concerning the question of transduction, Dr. Furth, just about 3 months ago, did report that in the recipients—animals injected at birth with leukemia extracts from a foreign strain—the genetic constitution of the leukemia appearing was frequently of the donor, indicating that the recipient had been changed genetically.

Dr. Syverton: I should like to comment on that point by introducing the concept of a specific inhibition of host response from injection within the first 24 hours of life of the unfiltered extract. This procedure should establish actively acquired tolerance in recipient animals as described and employed for other purposes by Medawar and his co-workers.

Dr. Gross: There are apparently some differences in technique in experiments carried out by Dr. Kirschbaum and his co-workers, and those originally performed in my laboratory. To begin with, Dr. Kirschbaum's group centrifuged the extracts for 20 minutes at 7,000×g, whereas we have carried out this centrifugation only for 5 minutes. Furthermore, Dr. Kirschbaum passed the centrifuged extracts through Seitz filters, whereas we have used Selas porcelain filter candles, or Berkefeld filters in our previous experiments. The Seitz filters contain asbestos pads which retain more than 90 per cent of many viruses. It is quite apparent, therefore, that Seitz filters are not suitable for filtration of leukemic extracts since they will probably retain most of the leukemic agent. Concerning the animals used for inoculation, it should be stated that there are differences in susceptibility which are related principally to the strains used, but also to other factors; thus, C3H mice of the Bittner substrain are more susceptible than those of the National Cancer Institute subline;

females appear to be more susceptible than males; foster nursed C3H mice (not carrying the mouse mammary carcinoma agent) appear to be more susceptible than C3H mice which carry the mouse mammary tumor agent. Concerning the incidence of spontaneous leukemia in untreated C3H mice of Bittner substrain in our laboratory, I can only state that this incidence in our experience is very low and does not exceed 1 per cent at the utmost. This is evident from data presented in our previous publications in which 92 out of 328 C3H mice inoculated with filtered leukemic extracts developed leukemia, whereas of 166 litter-mate controls injected simultaneously with heated (65 to 68° C. ½ hour) extracts only 2 developed leukemia (less than 1 per cent) and 5 developed parotid tumors; the remaining 158 control mice died negative at an average age of 16 months. In many other experiments, we have observed C3H mice through their life-span, many of them up to 24 months of age. We have not seen spontaneous leukemias beyond the incidence of 0.5 per cent to 1 per cent at the utmost. In view of the fact that Dr. Kirschbaum reports a higher incidence of spontaneous leukemia in C3H mice in his laboratory, it is possible that some of these leukemias may be reticular tumors of perhaps slightly different nature, and then again, there may be some other reasons for which I have no explanation at this time. The C3H mice are certainly not entirely free from spontaneous leukemia; as a matter of fact in a considerable number of such mice leukemia can be induced without the injection of an extract, only by total body x-ray irradiation or estrogenic hormones. It is quite apparent that C3H mice carry a latent leukemic factor. This latent factor may be distinct from the agent which we inject. There may exist different types of leukemia. This is pure speculation at this time, but it is entirely possible that such different types of leukemia may be caused by distinct agents.

Concerning Dr. Sabin's question: After we have found that filtered leukemic extracts prepared from leukemic Ak or C58 mice with spontaneous leukemia may induce luekemia following inoculation into newborn mice of a susceptible strain, it became apparent that the results of individual experiments may vary to a considerable degree depending upon the particular extract used. Some extracts were highly potent, other extracts were less potent, and some were entirely negative. After we have

selected a very potent extract, we have passed it through serial cell-free inoculations into successive generations of newborn mice. In each passage, the first mouse developing leukemia was used as a donor for the preparation of the next passage-extract. After several passages, we have succeeded in obtaining an extract of unusual high potency. Such extracts now induce leukemia in the majority of the inoculated mice after a brief incubation period of only 3 to 4 months.

Dr. Dmochowski: There has been a comparison made between some kinds of leukemias and mammary tumors. As one who has some little experience with this so-called Bittner virus, I would like to add to the comments that Dr. Kirschbaum has made. He says there are leukemias in about 15 strains carrying spontaneously. That is perfectly true, but in no way contradicts the viral origin of leukemias induced in the way Dr. Gross has induced them. I would like to ask Dr. Kirschbaum which of his low cancer strains or of his test mice, is entirely free of mammary tumors? That as such does not in any way disqualify the test animals derived from the low cancer strains. There may be different mammary tumors, as there may be different leukemias. Yet, there are mammary tumors which are induced by a virus. So, it would be of interest to know in Dr. Kirschbaum's case when he has induced or accelerated leukemia whether there was any leukemia in his litter control mice? That would be the deciding factor, not the appearance of leukemia at an old age which may be of a different character since using the comparison with mammary tumors there may be tumors which do occur at an old age which may not have the Bittner virus. It certainly may be so in the case of leukemia which occurs at an old age, and it may be of different origin.

Dr. Kirschbaum: Since a mammary tumor agent cannot be revealed in the mammary cancers which occur late, the question arises as to whether or not a virus had anything to do with the origin of these mammary cancers. If one obtains more mammary cancers by administering the agent, does one accelerate a process which is occurring spontaneously? Is the virus actually necessary? Years ago we induced mammary cancer with carcinogenic hydrocarbons, and during the last several years we have been assaying such tumors for agent, and I can say definitely that

no agent is revealed in these mammary tumors. Now is the carcinogen acting independently, or is a virus involved? These carcinogen-treated animals presumably have no agent, they have been foster-nursed by low-tumor females. We can induce mammary cancer in 60 to 80 per cent of them. Dr. Dmochowski himself has assayed such mammary cancers for agent, and has found none.

Dr. Woolley: The evidence from our studies would suggest that a number of workers might profitably explore the area of virus tumors in the mouse. It would further suggest that these studies should be done on a variety of genetic backgrounds; including both that of the virus source and of the receiving host. This should include not only strain differences but subline differences. Since the results seemed to depend so much on the particular preparation or filtrate used it would appear that much information could be obtained by the study of these filtrates individually rather than as pooled materials.

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* Formal discussion by:
George Woolley, Division of Steroid Biology, Sloan-Kettering Institute, New York 21, N.Y.
Noraki Ida, Section of Experimental Pediatrics, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston 25, Texas.
Arthur Kirschbaum, Anatomy Department, Baylor University College of Medicine, Houston 25, Texas.

ISOLATION AND IDENTIFICATION OF TUMOR VIRUSES

J. W. Beard*

A foremost problem in contemporary cancer research is that of determining the nature of viruses which cause tumors. While this has been a question of much interest for years in relation to growths long known to be of viral etiology, the vital necessity for concentration of attention on this field has been reemphasized by the increasing realization of the potentialities of viruses as specific carcinogens in the higher animals. That these agents are responsible for some malignant conditions has been known for half a century. The vital etiology of the avian leukemias was demonstrated in 1908 (Ellermann and Bang, 1908; Ellermann, 1921), and that of fowl sarcoma was reported in 1911 (Rous, 1911). In the following years, the number and kinds of virus-induced tumors of the chicken were increased to include the various forms of leukosis (Ellermann, 1921; Engelbreth-Holm, 1942; Jungherr, 1952), the category to which the leukemias belong, and many sarcomatous conditions (Claude and Murphy, 1933; Foulds, 1934) of varied pathologic aspects. For a time it seemed that such diseases were peculiar to the fowl. Extension of the problem to the mammal was achieved by the discovery of carcinomas in rabbits derived from papillomas caused by a virus (Rous and Beard, 1935) and, shortly afterward, of the association of a filterable agent (Bittner, 1936; 1946–47) with naturally occurring mouse mammary carcinoma. The significance of these findings with tumors in the mammal was greatly enhanced by observations (Gross, 1951; 1957; Wooley and Small, 1956) which have shown that a form of leukemia is transmissible by virus in *newborn* mice. More recently (Friend, 1956; 1957) another virus strain of leukemia has been found, which can be passed and is rapidly fatal in *adult* Swiss mice. Leukemia has

* From the Department of Surgery, Duke University School of Medicine, Durham, North Carolina. Preparation of this paper was aided by research grants to Duke University from the American Cancer Society, on recommendation of the Committee on Growth; from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; and by the Dorothy Beard Research Fund.

been induced in mice also by cell-free filtrates of several mouse tumors (Graffi *et al.*, 1956).

With all of these conditions, there has been a wealth of studies on pathogenesis, pathologic anatomy and on the factors influencing tumor occurrence. The results in all instances have seemed to stress the complexities of all of the diseases and of the interrelationships of varied associated manifestations. In the avian leukosis complex, figure I, for example, there are grouped (Beard, 1957a) the leukemias, myeloblastosis and erythroblastosis, together with the more prevalent conditions of lymphomatosis

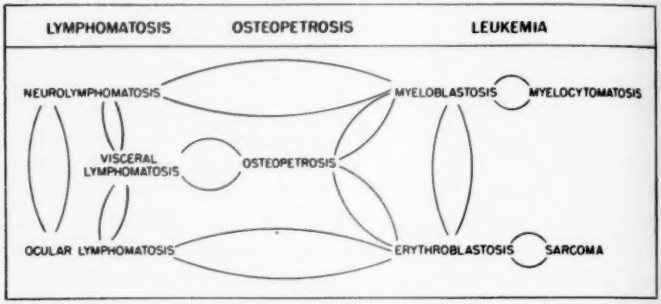


FIG. I. The major conditions of the avian leukosis complex. The connecting curves indicate, in part, the diversity of combinations occurring naturally or transmissible by experimental means.

(Burmester, 1952), the visceral, neural and ocular types, and osteopetrosis, which is not a lymphoid disease but one causing hyperplasia of the periosteum of the large bones. Many forms of avian sarcomas have been described (Claude and Murphy, 1933; Foulds, 1934). In rabbit papillomatosis (Shope, 1933), there is a progression from papillomatosis to outright carcinomatosis (Rous and Beard, 1935; Syverton, 1952) and, most confusing, the carcinomas, after repeated transplantations may lose all evidence of the presence of virus (Rous *et al.*, 1952). Some mouse mammary carcinomas contain no demonstrable virus, and occurrence of the growths is greatly influenced by hormones and genetic constitution of the hosts (Bittner, 1948). The understanding of the leukemias studied by Gross is confused by the appearance in mice after inoculation of virus, not only of leukemia, but of tumors of the parotid gland; sarcomas at the site

of injection; and of growths in the adrenal gland (Gross, 1953; 1956; 1957; Stewart, 1955). The leukemia found by Friend (1956; 1957) arose in a mouse bearing the Erhlich ascites tumor. All of these and many other findings have raised questions of interrelationships which have not yet been clarified.

A new method of study of now frequent application is that of the examination of ultrathin sections of cells with the electron microscope. Evidence has been obtained by this means of the association of abnormal intracellular particles with malignant cells. Much has been learned, also, about the morphologic aspect of the particles in the cells of growths known to be caused by virus and of the relationships of the particles to cellular structure. This approach has been of inestimable value in the demonstration of specific particles which have been recognized by no other means, as in the studies on the Rous and other chicken sarcomas (Gaylord, 1955; Haguenau *et al.*, 1955; Rouilles *et al.*, 1956) on the agent of mouse mammary cancer (Bernhard *et al.*, 1956; Dmochowski and Grey, 1957) and on the cells of mouse leukemia (Dmochowski and Grey, 1957). Particles with the appearance of virus have been observed in spleen cells of chickens with erythroblastosis (Benedetti *et al.*, 1956). Another and more significant problem has been raised, however, by the discovery of characteristic particles in the cells of growths for which there has been no evidence yet of viral etiology. Prominent in this respect are the structures seen (Selby *et al.*, 1954) in the cells of the Ehrlich mouse ascites tumor. Dalton and Felix (1956) have demonstrated particles in mouse tumors S37 and melanoma S91, which resemble the analogous structures found in chicken tumors. Some of the cells of spontaneous hepatomas of mice contain virus-like particles (Fawcett and Wilson, 1955), and particles have been reported (Rouilles *et al.*, 1956) in a chemically induced tumor of the chicken. The relation of such particles to the occurrence of the respective conditions remains obscure.

From what is already known it is clear that viruses are firmly implicated as carcinogenic agents, and it is likewise evident, from the rapid accumulation of information, that an understanding of the ultimate possibilities of their association with cancer is only now receiving general consideration. Furthermore, it can be seen that an intensive study of the nature of the agents and of their qualitative and quantitative relationships to the induction of

malignancy is of critical importance. A major approach to such studies is that of physical isolation of the agents. The advantages to be expected from access to preparations of the viruses in which they can be characterized and assayed is clearly attested by the results of similar studies on viruses not causing cancer and by the findings with the few tumor viruses already investigated by this means. The isolation and identification of tumor viruses are subject to numerous difficulties (Beard, 1957b; Bryan and Moloney, 1957) not arising with the ordinary agents. These are related principally to relatively long latent period of the diseases; profound genetic influences manifested in wide variations in host susceptibility and resistance; high median resistance of the host population; variation in the mass of virus from one tumor to another; and, finally, the relation of the physical mass of virus to the complexity of the tumor source material from which virus must be separated. Thus far only 3 tumor viruses have been isolated and characterized. These are responsible for rabbit papillomatosis (Bryan and Beard, 1941) and for the avian leukemias, myeloblastosis (Sharp *et al.*, 1952) and erythroblastosis (Bonar *et al.*, 1956). Many of the problems to be encountered and the means for circumventing them, as well as the kind of information to be gained, are well exemplified by the results of investigations of the leukemia viruses, which are the subjects of the present discussion.

The Avian Leukemias

These diseases of the chicken are elegant examples of the leukemic state. Myeloblastosis results from virus infection of the cells concerned with the origin of the *myelocytic series* of white blood cells (Furth, 1930; Engelbreth-Holm, 1942). It is a profound leukemia marked by the presence in the circulation of primitive cellular elements of the character of myeloblasts (Burmester, 1953), which reach levels of more than 2 million per mm^3 (Eckert *et al.*, 1954b). The condition is somewhat analogous to myelogenous leukemia in man. Erythroblastosis is concerned with the *erythroid elements* (Furth, 1931) and in manifested by the presence of primitive cells with the characters of erythroblasts in numbers up to several hundred thousand per mm^3 in the circulating blood. In both diseases the response to experimental virus infection is rapid (Eckert *et al.*, 1954a; Eckert

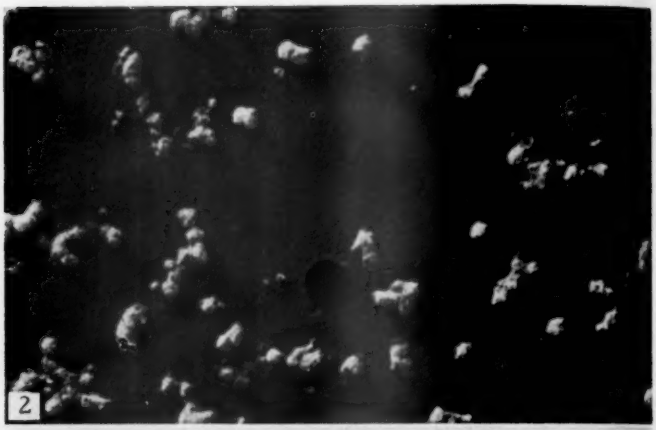
et al., 1956) and results quickly in death of the host. It is notable, here, that the various forms classified as lymphomatosis differ greatly from the leukemias in the infrequent occurrence of abnormal cells in the circulation (Burmester, 1952).

As will be indicated later, myeloblastosis and erythroblastosis are distinct pathologic entities, and, in addition, as shown by the results of investigations made possible by access to isolated virus, are caused by entirely different, though related agents (Beard *et al.*, 1957; Beard, 1957a). The myeloblastosis virus studied, the BAI strain A (Eckert *et al.*, 1951), was derived initially (Hall *et al.*, 1942) from cases of neurolymphomatosis but has been carried in the laboratory at Duke for years as a pure strain passed by the agent in filtered plasma or purified concentrates. The erythroblastosis virus was the Engelbreth-Holm strain "R" (Engelbreth-Holm, 1942; Eckert *et al.*, 1956) which, passed only with filtered material, has maintained consistent properties in this country since 1955. It is notable that the strain was developed originally from a case of *myeloid leukosis*.

Isolation and Physical Properties of the Viruses

It is well known that the viruses of the leukemias are present in the plasmas of birds with the respective diseases. This has been repeatedly demonstrated (Furth, 1930, 1931) by transmission of the conditions with filtered plasma, but until the present studies, there was no means for judging the *physical* concentration of the agents. The work was begun with myeloblastosis, and it was the intent to look for the virus in the plasma rather than in extracts of the malignant or other host cells. The first physical evidence of material of possible viral nature was the detection of particles (Beard *et al.*, 1950; Sharp *et al.*, 1952) in filtered plasma from affected birds which were not seen in like fluid from normal chicks. These particles, which later proved to be the agent (Beard *et al.*, 1955), were approximately spheroidal in shape and variable in size as seen in the lectron micrograph of figure II. Their average diameter in these pictures was about 120 mμ. An outstanding problem encountered at once was evidence of extreme distortion of the particles when they were dried on collodion in the presence of salt. Preparations obtained by drying the particles from distilled water yielded no pictures at all. The difficulty was overcome after a time, however, when a

method was devised (Sharp *et al.*, 1952) for drying the particles on an agar surface which resulted in the simultaneous diffusion of both salt and water into the agar. The particles could then be fixed with osmic acid vapor, lifted off the agar in a collodion film and shadow-cast for electron micrography.



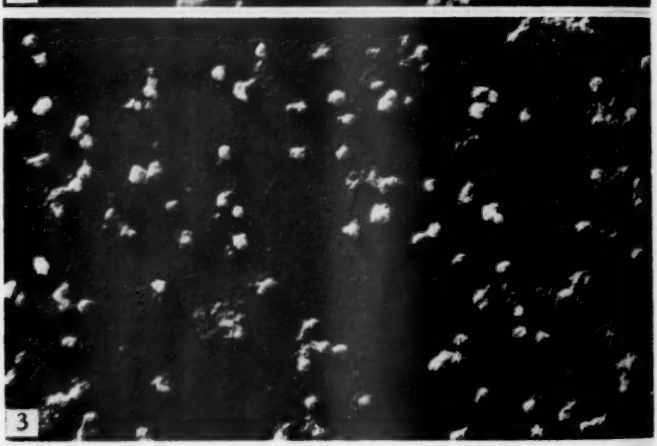


FIG. 2. Myeloblastosis virus in a purified preparation. Magnification 27,000 X.

FIG. 3. Erythroblastosis virus in a concentrate from plasmas of chickens with the disease. Magnification 27,000 X.

A finding of much significance, both for the present work and for an understanding of the principles concerned with isolation of other tumor viruses, was an extreme variation in the concentration of the particles in the plasma (Eckert *et al.*, 1952; Mommaerts *et al.*, 1954). In order to measure the variation, a method was developed for counting the particles with considerable accuracy. This was accomplished by sedimenting the particles from a measured volume of plasma and counting them directly in the electron microscope (Sharp and Beard, 1952). By this means it was found that the greatest concentration was of the order of 2 trillion per ml. of plasma (Mommaerts *et al.*, 1954; Sharp *et al.*, 1954; Eckert *et al.*, 1954b), with a variation to less than the limit of the method of estimation, namely, about 1 billion per ml. That the levels were as low as a few thousand particles per ml. was indicated by the fact, as will be seen, that the plasmas from some birds dying of the disease were noninfectious (Eckert *et al.*, 1952) when injected into susceptible birds. This phenomenon of extreme differences in concentration of virus associated with tumors of viral etiology is of critical importance in the total problem of isolation and study and has been encountered with all such growths, the chicken sarcomas (Bryan, 1955), rabbit papillomatosis (Beard, 1956), and mouse mammary carcinoma, as well as the forms of avian leukosis. The findings are particularly striking with myeloblastosis because of the variety of methods affording quantitative studies of virus amount.

In its physical structure the virus of myeloblastosis differs greatly from most other filterable agents. The extreme distortion of the particles (Beard *et al.*, 1950; Sharp *et al.*, 1952) drying in the presence of salt suggests a loose watery structure which was confirmed by the value 1.059 for hydrated density determined by sedimentation in bovine serum albumin (Sharp and Beard, 1954). The average diameter of the particles, estimated by rate of sedimentation, was 144 mμ. Examination of the particles lightly shadowed with chromium revealed the presence of a limiting membrane enclosing a centrally located spherical, nucleus-like structure of high election absorbing power, resembling analogous material associated with other viruses.

The erythroblastosis virus is morphologically indistinguishable, figure III, from that of myeloblastosis by electron microg-

raphy. In the pictures thus far obtained, the former has seemed slightly smaller than the virus of myeloblastosis.

Isolation of the myeloblastosis virus is relatively easy because of the simplicity of the suspending medium and high concentration of the virus (Beard, 1957b). Suitable plasmas, selected as described in a later section, are filtered with celite, passed through a bacterial filter, and the virus is sedimented by ultracentrifugation at 20,000 x g for 45 minutes. After resuspension of the pellets in saline solution, the virus is sedimented again as before. A third cycle may be employed, but the particles tend to aggregate increasingly after the second sedimentation (Sharp and Buckingham, 1956). Amounts of about 1 mg. of virus can be recovered from 1 ml. plasma. The principle of purification consists, simply, in washing away the low molecular weight proteins of the plasma. The level of purity or homogeneity attained is related to the concentration of virus particles in the source material (Beard, 1957b). Plasmas of normal chickens, and presumably those with myeloblastosis, contain about 1 billion particles per ml., which come down when the virus is sedimented. However, if the concentration of virus particles is of the order of 1 trillion per ml., as is the case, the sedimented material will consist, approximately, of 99, or more, per cent virus. This is illustrated in figure IV which indicates the proportion of virus to extraneous material to be obtained from plamas containing different virus amounts in relation to constant content of non-viral material.

The dependence of successful results on virus mass is well illustrated by the findings with erythroblastosis in comparison with those observed with myeloblastosis. As stated before, the level of erythroblastosis virus in plasma is seldom as high as 100 billion per ml. in the best chickens. Since there is no convenient means for selecting plasmas of highest virus content, isolation procedures must be applied blindly, usually to large plasma pools containing about 10 billion particles per ml. In consequence the concentrated product will consist of virus with a large proportion of extraneous particles, as shown in figure III, which cannot be eliminated by centrifugal fractionation (see Fig. IV). Because of the low virus concentrations, it has not been possible to obtain purified preparations; indeed, the amount of extraneous material in the concentrates interferes substantially with quantitative

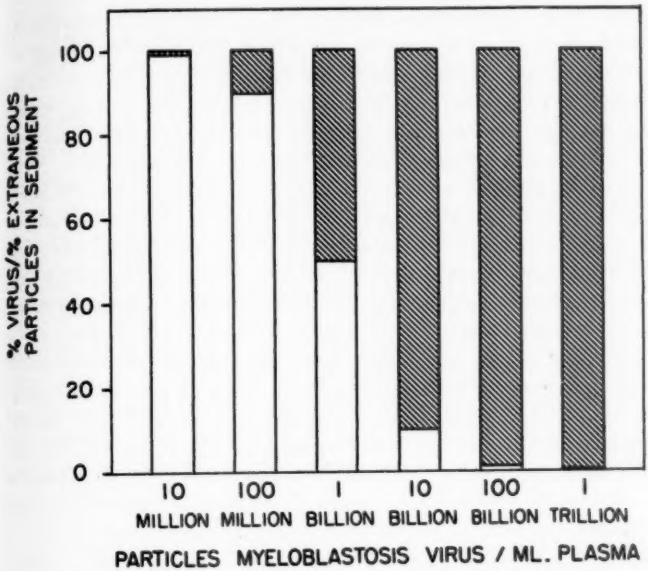


FIG. IV. The ratio of virus (diagonal shading) to extraneous material sedimented from plasmas of chickens with myeloblastosis containing about 1 billion non-viral particles in relation to different concentrations of virus in such plasmas.

enumeration of the virus particles. Electron micrographs have shown in a few preparations, however, that the agent of erythroblastosis is closely similar in its physical properties to the virus of myeloblastosis. Both agents exhibit like regions of pH stability, but the virus of erythroblastosis is much the more stable on storage (Eckert *et al.*, 1955b; Eckert *et al.*, 1955c; Bonar *et al.*, 1957a).

Enzyme Activity

Progress in all fields of the investigation of viruses is wholly dependent, in final analysis, on methods for quantitative assay of the biological activities of the respective agents. This has accounted largely for the upsurge in work with bacteriophages which can be bioassayed by infectivity measurements cheaply and in a matter of a few hours. A similar advantage is experienced with the influenza virus and some of the other agents of

animal disease which can be assayed rapidly by the hemagglutination reaction. Many other animal viruses are "highly infectious," and effective experiments can be made by estimates of infectivity. The tumor viruses, in contrast, can be titrated, under the best of circumstances, only by tedious, very expensive and time consuming efforts, which will be discussed later.

An endowment of most vital consequence is the enzymatic activity of the virus of myeloblastosis to dephosphorylate adenosine and inosine (Green and Beard, 1955b) triphosphates. This phenomenon, discovered (Mommaerts *et al.*, 1952) early in the work with the virus, is a property thus far unique, among viruses, to the myeloblastosis agent. The reaction is pronounced (Green and Beard, 1955a), and is directly and quantitatively related, figure V, to the number or mass of virus particles (Mommaerts *et al.*, 1954; Sharp *et al.*, 1954; Eckert *et al.*, 1954b). Virus amount can be estimated with much accuracy by methods involving determination of phosphorus split away from adenosine triphosphate (Mommaerts *et al.*, 1954) or of the phosphoric acid

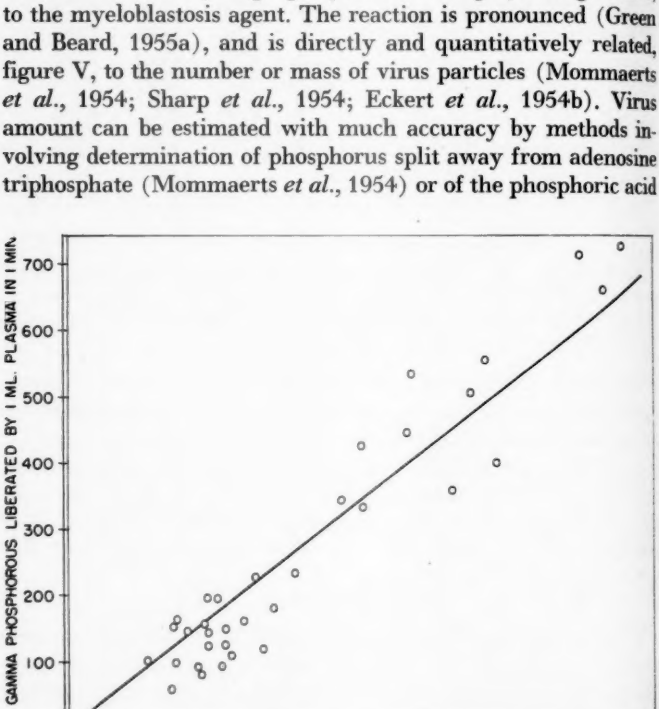


FIG. V. Relation of phosphorus liberated from adenosine triphosphate to the number of virus particles in plasmas from chickens with myeloblastosis.

resulting from hydrolysis (Green and Beard, 1955a). The latter reaction has been made the basis for a micro method (Mommaerts *et al.*, 1953; Green *et al.*, 1954) suitable for the rapid screening of hundreds of diseased chicks for selection of those of high virus concentration suitable for isolation and other procedures. The test can be applied repeatedly to very small chicks since only 3λ (0.003 ml.) of plasma are required for an accurate result. The reaction has served as a major method for virus detection, estimation, and correlation with other properties, and has brought within reach a vast field of investigations which would not be practical to undertake with any other method now recognized. The enzyme activity is relatively stable (Eckert *et al.*, 1955c) in comparison with the property of infectivity and, of much interest, is not lost or decreased when virus activity is neutralized with immune serums (Eckert *et al.*, 1955d). Results obtained in the procedures for identification of the virus have revealed good evidence that the enzyme activity is an intrinsic component of this virus particle.

In striking contrast to this activity of the agent of myeloblastosis is the inconsequential, if any, activity of the erythroblastosis virus to hydrolyze adenosine triphosphate (Bonar *et al.*, 1957b). Plasmas from chickens with erythroblastosis are somewhat more active in the hydrolysis of adenosine triphosphate than those from normal birds, but little of it can be ascribed to the virus. No erythroblastosis plasma has been observed to yield a reaction in the micro screening test (Eckert *et al.*, 1956). This criterion serves at once to distinguish myeloblastosis virus from that of erythroblastosis, an extremely significant difference, inasmuch as it is now known that the two agents are immunologically closely related (Beard *et al.*, 1957) and are members of a family (Beard, 1957a) responsible for tumors of the chicken of distinct pathologic manifestations.

Antigenic and Immunologic Properties

Clarification of numerous questions of the interrelationships of the various forms of avian leukosis in years past has been hindered by the lack of specific immune bodies. Although much work has been attempted in the field (Olson, 1940; Jungherr, 1952), little has come of it because of the confusing and contradictory results of different investigators. With adequate amounts

of antigenic material provided by the virus-rich plasmas of birds with myeloblastosis, it is no more difficult to induce immune bodies in the chicken, rabbit or other heterologous hosts than with other viruses (Eckert *et al.*, 1955d). Antibodies can be produced in the chicken with concentrates of the virus inactivated with formalin; or older birds, highly resistant to infection with the agent, can be immunized with the active virus in plasma. Antibodies obtained in this way are very effective in neutralizing virus infectivity and in precipitation of the virus as can be seen, macroscopically, by aggregation and settling of the flocs in the test tube. The results of precipitation can be demonstrated beautifully by electron micrography of the aggregates, figure VI, deposited on agar (Eckert *et al.*, 1955d; Beard *et al.*, 1957).

The most interesting reactions were obtained (Eckert *et al.*, 1955d) with immune serums from rabbits injected with virus concentrated and purified by ultracentrifugation. The rabbit anti-viral antibodies fixed complement with the virus concentrates and, in addition, neutralized the infectious properties. A similar and, at the time, disturbing observation was the activity of the anti-viral antibodies to fix complement also with normal chicken tissue. Because of the uncertainty of the purity or homogeneity of the virus preparations at that stage, the results seemed related most probably to chicken protein not eliminated in the purification process. This possibility was emphasized by demonstration of the activity of the serum of rabbits injected with normal chicken serum or tissue (spleen and liver) to fix complement with the virus concentrate. Many efforts were made to apply various procedures designed to separate the virus from hypothetical chicken protein, but all showed that reduction of chicken tissue antigenicity resulted in a comparable diminution in the virus content of the preparation.

Failure in this direction led to diversion of attention to the possibility that the chicken antigen was either very intimately bound to the virus particles or was an integral part of them. The entire problem was resolved simply and effectively by investigation of the seemingly unlikely possibility that the virus could be neutralized by anti-normal chicken protein immune serum from the rabbit. Contrary to what had been expected, it was found that such anti-serums were quite active in the neutralization reaction. Another property of the virus concentrates was their

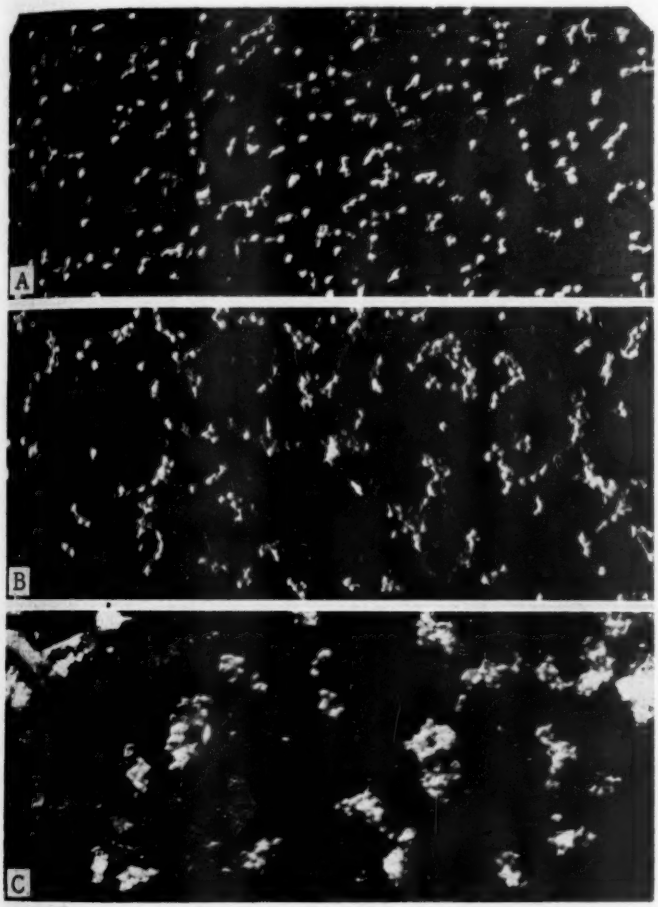


FIG. VI. A. Myeloblastosis virus particles in filtered plasma. Magnification 13,800 X.

B. Specific aggregation of the particles of the plasma of A occurring within 1 minute after treatment with anti-myeloblastosis immune serum from the chicken. Magnification 13,800 X.

C. Specific aggregation of the particles of the plasma of A occurring within 3 minutes after treatment with anti-myeloblastosis immune serum from the chicken. Magnification 13,800 X.

demonstrable content of Forssman antigen. Suitable experiments showed that Forssman antibodies from rabbits injected with guinea pig kidney likewise neutralized the virus, though not as well as specific antibodies from the chicken or those, including anti-chicken tissue immune bodies, from the rabbit. The implication of the total findings, which are summarized in figure VII, are discussed in the section concerned with identification.

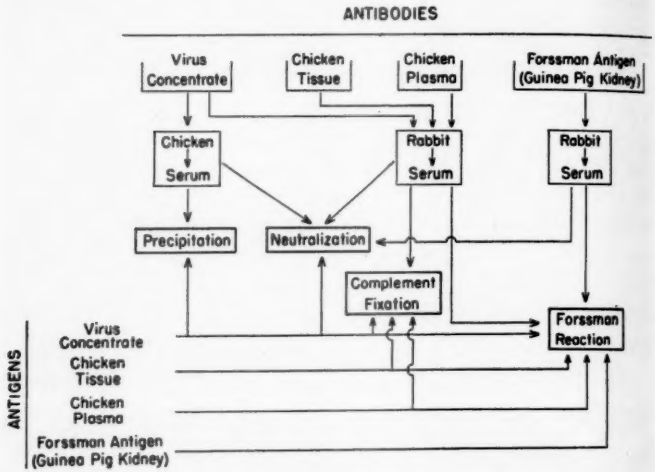


FIG. VII. Diagram of the reactions and cross reactions between virus concentrates, the chicken antigens of chicken plasma (which reacts in the same manner as normal chicken serum), the chicken antigens of chicken tissue, and the Forssman antigen (guinea pig kidney) with the respective serums induced in the chicken and in the rabbit.

With the considerable background of knowledge of the myeloblastosis virus, analogous studies were carried out with erythroblastosis. Here the problem was greatly hampered by the small amounts of the agent available. An essential reagent was an anti-erythroblastosis immune serum from the chicken. Enough virus for the immunization of 8 chickens was obtained, however, by ultracentrifugation of more than 2 liters of plasma taken from 200 chickens. Inoculation of the concentrate inactivated with formalin produced immune serum which neutralized the agent (Beard *et al.*, 1957) and produced a precipitation reaction which was demonstrable by electron micrography (Bonar *et al.*, 1956).

This reaction has been the sole means for unequivocal identification of the agent.

As in the studies on myeloblastosis, it was observed that the erythroblastosis virus was neutralized (Beard *et al.*, 1957) by anti-chicken protein immune serums from the rabbit. In this respect, then, the two viruses were similar in antigenic behavior. A pronounced difference was seen in the failure of Forssman antibodies to neutralize the erythroblastosis virus.

Identification and Correlation of Properties

It is not often difficult, now, to recognize viruses in their physical form. This was not true in the early days of study, during the latter years of the 1930's. Then, there were available only indirect methods, chiefly analytical ultracentrifugation for study of sedimenting boundaries and electrophoresis for observation of the migration of materials in an electrical field (Beard, 1948; Sharp, 1953). These and other procedures gave some information about the possible size and shape of the entities, and chemical analysis furnished a notion of their constitution. The whole perspective of the problem was changed with the development of the electron microscope which became available in practice from about 1940. With this instrument, viruses could be seen, and pictures could be made (Bergold, 1953; Williams, 1954). In the electron micrographs, it could be demonstrated that viruses were particles of various sizes and forms fairly easily distinguishable from host materials. A notable exception has been the poliovirus, the recognition (Bachrach and Schwerdt, 1954) of which has required many years of study.

The virus of myeloblastosis, however, presented unusual problems. Occurrence of particles of uniform morphology in high concentration in the plasma of some diseased birds and their absence in normal ones might have been acceptable as presumptive evidence of the identity of the virus with the particles. A different light was thrown on the matter, however, with the discovery of the enzymatic activity of the plasmas, and further difficulties were introduced by the presence of normal chicken antigen in purified concentrates of the agent. Enzymatic activity to hydrolyze adenosine triphosphate and a vital association of host antigens were properties unique to any viral agent then

studied. It was evident that ultracentrifugal concentrates of the plasma from diseased birds exhibited five major characteristics: they contained particles of uniform morphology; induced the infection process; hydrolyzed adenosine and inosine triphosphates (Green and Beard, 1955b); behaved immunologically as host antigen; and contained Forssman antigen. It then became essential either to effect a separation of the materials responsible for the various activities or to produce unequivocal evidence that all of the properties were those of the virus itself.

Fortunately, as it turned out, the question was readily resolved (Mommaerts *et al.*, 1954; Sharp *et al.*, 1954; Eckert *et al.*, 1954b; Eckert *et al.*, 1955b) by virtue of the peculiar behavior of the agent which had caused so much confusion in the first place. The initial studies were concerned with attempts to separate the different properties by ultracentrifugation. When refinement of the purification procedure was without effect, investigations were made to compare quantitatively the rates of sedimentation of the respective activities. By this means it was learned that the materials causing infection, enzyme activity and acting as chick and Forssman antigens came down at the same rate as did the particles, which could be followed quantitatively by the counting procedure already described. Analogous experiments with electrophoresis indicated that the particles and the various activities migrated at the same rate in an electrical field. Inasmuch as the findings provided a good correlation by three wholly unrelated principles; morphology, as seen in the electron microscope; size, shape and density, which determine rate of sedimentation; and rate of migration by electrophoresis, which is related to electrical charge on the particle; it could be judged with considerable assurance that all of the properties were those of the particles and, consequently, of the virus.

Unequivocal identification and correlation were established finally by immunologic procedures (Eckert *et al.*, 1955d). It had been learned that the particles could be precipitated quantitatively and infectious activity neutralized by specific immune serum from chickens. The characters of the aggregated particles could be seen in electron micrographs, figure VI, and, examination revealed a proportional lessening in the number of the particles remaining in suspension. As noted before, the enzymatic activity of the virus is not diminished by the action of im-

mune serum. This provided an elegant means for correlating the particles with the enzyme. When the particles were precipitated with immune serum, the capacity to dephosphorylate adenosine triphosphate was found associated with the aggregated material and had been removed from the suspending fluid. The precipitation reaction was useful also in demonstrating the relation of the particles to host and Forssman antigenicity. The particles aggregated by immune serum, sedimented, and washed, retained the capacity to fix complement with anti-normal host immune serums from the rabbit and likewise gave the Forssman reaction with anti-guinea pig tissue immune bodies.

The really critical evidence of association of normal host protein and Forssman antigen with the virus particles obviously was the neutralizing effect of the heterologous serums from the rabbit (Eckert *et al.*, 1955d). This reaction occurs on incubation of mixtures of the rabbit immune serums with virus in the *absence* of complement. Earlier work by several investigators had given evidence that the virus of the Rous sarcoma may be neutralized in an analogous way but only in the *presence* of complement in the mixtures (Gye and Purdy, 1931; Amies and Carr, 1939). The results of recent work with this virus have suggested (Rubin, 1956) that the action of the anti-normal host protein immune bodies is reversible on dilution of the mixtures and is not on the virus, but directly on the host cells. Experiments similar to these, within the limits of the conditions applicable, revealed entirely different results with the erythroblastosis virus (Beard and Beard, 1957). The findings indicated that neutralization by heterologous immune serums does not differ in principle from those by homologous antiserums and that the activity is directly on the virus and is not reversible on dilution. There still remains no evidence contrary to the hypothesis that the virus particle contains, as intrinsic components, (a) specific virus antigen effective in the chick; (b) material antigenically identical with normal host protein; and (c) Forssman antigen.

Interrelationships of Myeloblastosis and Erythroblastosis Viruses

The etiologic interrelationships of the various diseases classified as members of the avian leukosis complex, indicated in figure I, have been a question of much concern (Beard, 1957a).

Earlier studies have shown that (1) each condition is a distinct pathologic entity which may, and does, occur individually; (2) any two or more may be found together under natural conditions; various mixtures can be propagated by cell transplants or by virus transmission with filtered material (Johnson, 1941); and finally, and most confusing, some of the diseases, particularly the leukemias, may be derived from birds with some other form of leukosis but showing no evidence of the derivative condition (Hall *et al.*, 1941; Engelbreth-Holm, 1942). Some investigators have supported the unitarian view (Johnson, 1941) which conceives the diseases as being of common etiology. This has been especially the case with the leukemias, which have been thought to be due to infection by a single virus (Engelbreth-Holm, 1942), the occurrence of myeloblastosis or erythroblastosis depending only on the type of cell parasitized. It has already been noted that the strain of myeloblastosis under study was derived from cases of *neurolymphomatosis* (Hall *et al.*, 1941) and that erythroblastosis came from a chicken with myeloid disease (Engelbreth-Holm, 1942).

Some aspects of this question have now been clarified satisfactorily, and a continuation to complete resolution is but a matter of time and application to the problem. In the present work it has been demonstrated that the leukemia viruses are certainly distinct entities, as seen by the striking difference in adenosine triphosphatase activity of the two agents (Bonar *et al.*, 1957b). A difference is seen again in the absence of Forssman antigen from the erythroblastosis virus (Beard *et al.*, 1957). These differences distinguish the agents unequivocally one from the other.

Immunologic investigations, however, have indicated a close antigenic relationship between the agents (Beard *et al.*, 1957). Anti-myeloblastosis immune serum from the chicken is effective in the neutralization not only of the homologous virus but of the erythroblastosis virus as well. The respective cross reactions worked equally well in the other direction. It will be recalled, further, that both viruses behaved as if they contained normal host antigen. The conditions of the experiments were not of a sort to permit recognition of quantitative differences.

Further information was obtained by precipitation experiments with anti-viral chicken immune serums (Beard *et al.*, 1957). Anti-myeloblastosis serum precipitated the homologous

virus quantitatively, as illustrated in figure VIII. The anti-erythroblastosis serum under identical conditions in the same experiment induced, figure IX, partial precipitation of the erythroblastosis virus. In cross reactions, figure X, anti-myelosblastosis serum produced partial, but good, precipitation of the erythro-

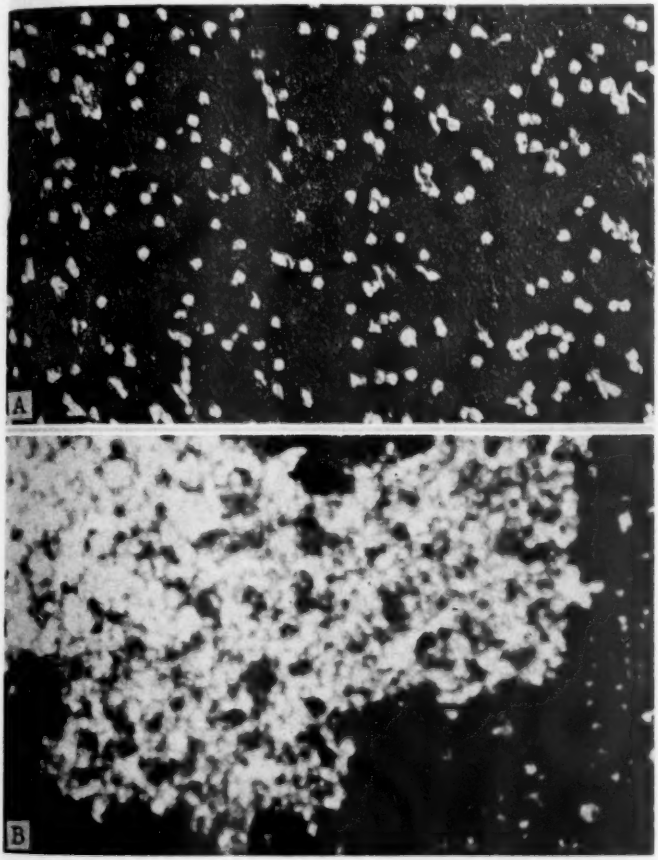


FIG. VIII. A. Virus of myeloblastosis in filtered plasma. Magnification 21,000 X.

B. Specific aggregation of the virus of myeloblastosis in filtered plasma after treatment with anti-myeloblastosis immune serum from the chicken. Magnification 21,000 X.

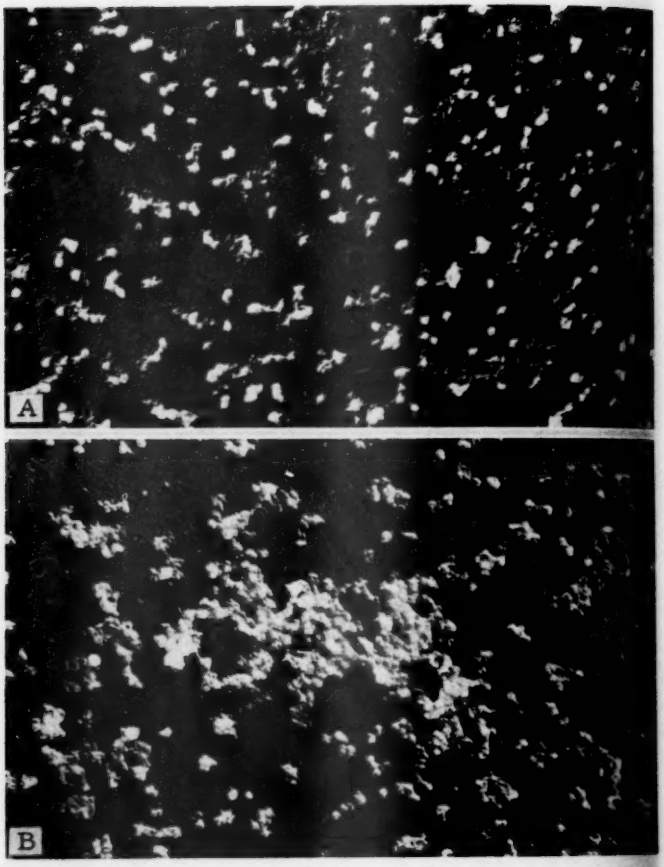


FIG. IX. A. Concentrate of erythroblastosis virus. Magnification 15,100 X.

B. Specific aggregation of particles in a concentrate of erythroblastosis virus treated with anti-erythroblastosis immune serum from the chicken. Magnification 15,100 X.

blastosis virus, but the anti-erythroblastosis serum had only dubious, if any, effect at all on the virus of myeloblastosis in concentrates in which all of the virus was aggregated by the homologous immune serum (compare with Fig. VIII). These experiments reveal quite clearly, on the one hand, that the agents are

specifically related in antigenic constitution but, on the other, that they are certainly not identical.

Although the data provide a sound basis for both differentiating and relating the leukemia agents, the full measure of kinship

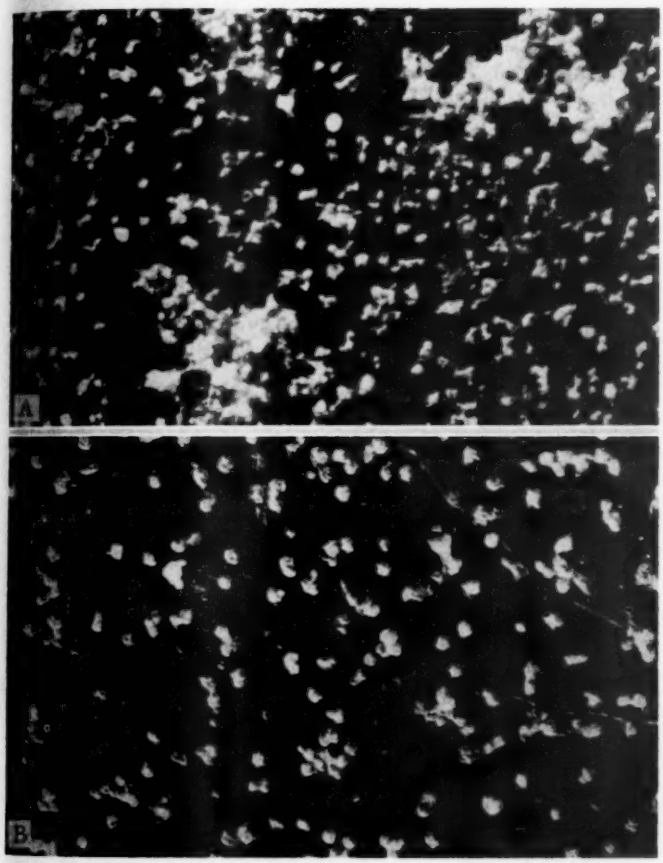


FIG. X. A. Partial aggregation of particles in a concentrate of erythroblastosis virus treated with anti-myeloblastosis immune serum from the chicken. Magnification 21,000 X.

B. Questionable aggregation of the virus of myeloblastosis in filtered plasma treated with anti-erythroblastosis immune serum from the chicken. Magnification 21,000 X.

is still obscure. It would seem that they constitute, in effect, "sister" viruses belonging to a distinct group of tumor viruses (Beard, 1957a). Questions may be raised relative to a common origin; to reversible transformation; or to transformation from one agent to another in a single direction. On the basis of the results of collaborative studies with Dr. B. R. Burmester of the Regional Poultry Research Laboratory, East Lansing, Michigan, and Dr. W. Ray Bryan, National Cancer Institute, a foundation has been established (Beard, 1957a) for relating the agents of the leukemias with the virus of lymphomatosis and with that of the Rous sarcoma. Inasmuch as all of the chicken sarcoma viruses thus far studied in this respect are immunologically related (Andrewes, 1931; 1933; 1934), there is some reason to suspect that the agents of the leukemias, of visceral lymphomatosis and of the avian sarcomas may constitute a family of interrelated viruses. It is well to note that visceral lymphomatosis is a contagious disease (Burmester, 1957), whereas there is no evidence of transmission by contagion of either the leukemias or of the many varieties of avian sarcoma. There exists the possibility for further study that the agent of visceral lymphomatosis may represent the parent virus in nature, derivative variants of which may be responsible for the virus-induced malignancies of the chicken.

Host Response

An informative outcome of studies with isolated tumor viruses has been the understanding of the quantitative relationships between the host and the virus in the induction of malignancy. Thus, much has been learned about the participation of the host in the reaction which can be measured in terms of the number of particles or mass of virus involved. Similar results have been obtained with both myeloblastosis and erythroblastosis, and the findings with the former will serve to illustrate the principles involved.

In figure XI there are shown the data obtained in a titration experiment with 6 successive 10-fold dilutions of virus injected intravenously in 0.1 ml. volumes into 30 chicks per dose group (Eckert *et al.*, 1954c). The experiment of figure XI was made in 3-day-old chicks of a relatively highly susceptible inbred strain of White Leghorns (Line 15, Regional Poultry Research Laboratory, East Lansing, Michigan). Host response to the agent is esti-

mated in terms of the relation of dose to the latent period of the disease detected by the appearance of primitive myeloblasts in the circulation (Eckert *et al.*, 1954a). The data of figure XI represent the accumulated number of chickens positive with each virus dose, expressed as probits, plotted against log latent period. The graph shows a linear first limb indicating a normal distribution of the states of resistance and susceptibility of the individual chickens to a given dose as estimated by differences in log latent period. Each curve deviates from the initial relation which has been regarded as evidence of additional resistance acquired during the experimental period following injection of virus. The graph shows the basic data from which calculations are made of the response to each dose of virus.

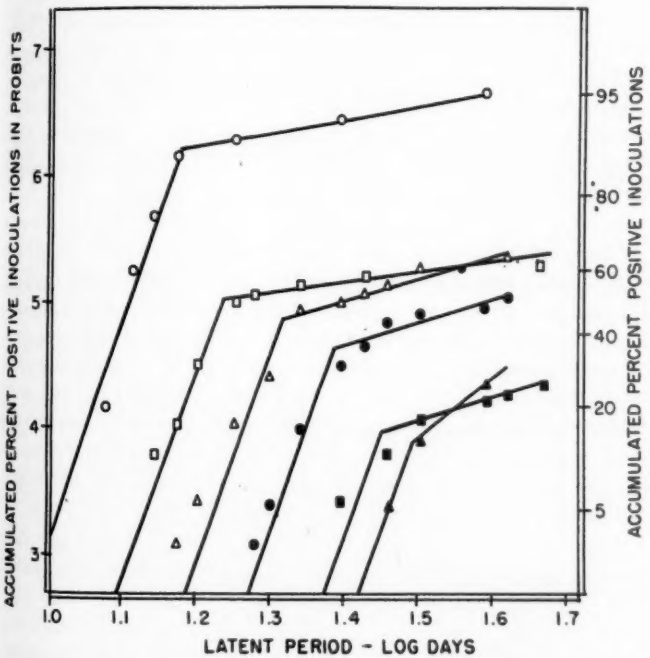


FIG. XI. Time-frequency distribution of host response of 3-day-old chicks to a series of six tenfold dilutions of plasma containing the virus of myeloblastosis (see experiment 124, table 1 of Eckert *et al.*, 1955a).

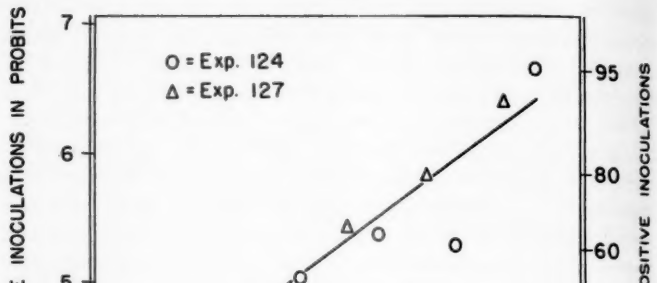


FIG. XII. Response of 3-day-old chicks to graded doses (numbers of virus particles) of the virus of avian myeloblastosis. The data for construction of the graph were given in table 1 of Eckert *et al.*, 1955a. Incidence was taken as total per cent positive inoculations.

The quantitative relations between number of virus particles and chicken response are shown in figure XII (Beard, 1956). In the abscissa there are tabulated the number of virus particles per dose and in the ordinate there is given, in probits, the incidence of chickens positive with each dose. It is seen that the median effective dose, that is, the number of particles required to cause disease in 50 per cent of the chickens inoculated (at the level of probit 5), was about 26 million. This constitutes a measure of the enormous median effective resistance of the population of chickens despite their relatively high susceptibility. Extrapolation of the linear relation of figure XII to 5 per cent level of incidence yields the value of approximately 6,000 particles required to infect this proportion of birds. This is in contrast with the number of about 145 billion particles which are essential to infect about 95 per cent of the chicks. These data are an index of the very broad variation in individual host susceptibility, which extends over the range of doses from 6,000 to 145

billion particles (24 million-fold) causing infection in chicks in the region between 5 and 95 per cent positive.

The findings with erythroblastosis were similar in principle to those with myeloblastosis except that erythroblastosis was from 100 to 1,000 times more virulent or infectious (on a particle number basis) (Eckert *et al.*, 1956) than myeloblastosis. Similar measurements have been made with the virus of rabbit papillomatosis with which the known range in individual host resistance is about 1000-fold, and the median effective resistance of the population is measured by the number of about 80 million virus particles necessary to cause infection in 50 per cent of the rabbits inoculated (Bryan and Beard, 1940). No estimates of similar nature can be made with other tumor viruses until the means becomes available for counting virus-particle numbers.

Another outstanding difference between the agents of myeloblastosis and erythroblastosis is concerned with the influence on host response of the age of the chicken at the time of inoculation. With both viruses, the response is highest when the agents are injected intravenously (Eckert *et al.*, 1955a; Beaudreau *et al.*, 1956). Under these conditions, chicks 3 days of age are much more susceptible to myeloblastosis virus than older ones (Eckert *et al.*, 1955a). In contrast, susceptibility to erythroblastosis virus increases greatly with increase in the age of the host (Beaudreau *et al.*, 1956). Host response to both agents is vastly affected by genetic factors associated with strain and breed difference in various chicken populations (Eckert *et al.*, 1954c; 1956). One strain of inbred White Leghorn chickens, however, has been of sufficiently high susceptibility to both leukemia viruses to permit quantitative measurements of infectious capacity. This is the line 15 developed at the Regional Poultry Research Laboratory at East Lansing, Michigan (Waters, 1945; 1951). Even with these chickens the difficulties of titration are great because of high median population resistance and of extreme variation in individual resistance and susceptibility. For every dose group, 30 chicks are required, and 20,000 birds are employed for routine studies each year. These factors are of still greater influence in the study of lymphomatosis which exhibits a much longer latent period (Burmester, 1956). Similar difficulties are experienced with the rabbit papilloma virus (Bryan and Beard,

1941) and the agent of the Rous sarcoma (Bryan, 1955; 1956; 1957; Bryan and Moloney, 1957).

Comment

It has been the intention in the foregoing discussion to indicate some of the findings encountered in studies on the isolation and identification of tumor viruses. A principal purpose of the account has been the illustration of the feasibility of the application of well recognized methods and procedures developed in analogous studies with other filterable viruses. Further, there has been emphasized the kinds of results which can be expected from access to the isolated agents. With such materials, many experiments otherwise unproductive become a matter of routine study, and obscure relationships are readily clarified. It cannot be assumed that the immediate procedures will be applicable in the same way to all of the known tumor viruses. Nevertheless, the results provide the basis and the stimulus for increased attention to a field long neglected.

Discussion

Dr. Gross: What is the relative size of the particles?

Dr. Beard: Dr. Gross has asked about the relative sizes of the particles inside cells and outside—there is no difference. Within the limits of our ability to measure the diameter of the material inside the cell and outside there isn't any difference.

Dr. Benedict, Galveston, Texas: Working with chickens one can do a direct precipitant test using chicken serum using chicken antibody. This may facilitate, or give a more sensitive method than the precipitant action. This is using unheated chicken serum with a suitable antigen.

Dr. Carter, Galveston, Texas: I would like to ask Dr. Beard if he has done any neutralization tests on chickens which failed to come down with this disease, after inoculation, not those which have been hyper-immunized.

Dr. Beard: No, we haven't done that. The study of leukosis has been very greatly confused and hampered in the past by the lack of good immune bodies. If one injects virus into a bird with a

dose calculated to bring it down, there will be little immunibody formed. A great deal of the virus is required to produce immune bodies. One might wonder why birds with the disease, with large amounts of virus, did not have immune bodies. That's not too difficult to answer. The birds which have enough virus in the plasma to produce immune bodies die right off. The birds that live long enough to produce immune bodies don't have enough virus in the plasma to produce antibody in the first place. It takes quite a bit of antigen in these birds to produce enough immune body to be measured by reasonable methods.

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BERTNER FOUNDATION LECTURE

STUDIES ON MAMMARY CANCER IN MICE AND THEIR IMPLICATIONS FOR THE HUMAN PROBLEM*

JOHN J. BITTNER†

After it was determined that some maternal influence was involved in the development of mammary cancer in mice (Staff, Jackson Memorial Laboratory, 1933) and following the demonstration of the milk-borne mammary tumor agent (MTA) in 1936 (Bittner), several articles appeared dealing with the possible significance and implications of the experimental data to the problem in humans. For this presentation it has been suggested that we examine the animal data, keeping in mind that the inbred strains of mice and the various hybrids might each correspond to different individuals, to indicate what we might have to know about the disease in humans before we may state, perhaps, that "man is or is not an overgrown mouse." In doing so, it will become obvious that certain of the observations obtained in mice could not be explained on the basis of any "bottle-feeding" theory, and one should not expect otherwise for humans, where the validity of the "experimental data" may be influenced by the wording of a question. That is, the answer to: "Were you breast-fed or bottle-fed?" might be quite different than that to the same question, worded: "How long were you breast-fed?"‡ Also, as new studies are completed and confirmed, we must be prepared to modify any theory, and, perhaps, the interpretation of earlier observations.

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† George Chase Christian Professor of Cancer Research and Director, Division of Cancer Biology, Department of Pathology, University of Minnesota Medical School, Minneapolis 14, Minnesota. The author wishes to acknowledge the valuable contributions of many associates, and especially the technical assistance of Ruth O. Vrudny and Dorothy V. McGrath.

‡ In view of what we know about the development of mammary cancer in mice, one breast-feeding would be adequate to transfer an agent.

JOHN J. BITTNER

The MTA has the characteristics of an infectious agent or virus, but we shall be concerned primarily with the biological aspects of the agent, and reference to the many investigations on the MTA may be found in the general review articles listed below.

The usual type of mammary cancer in mice results from the action of several causative factors: hormonal stimulation, the MTA, genetic factors, etc. (Bittner, 1939). The significance of each in the genesis of these tumors was emphasized by Heston (1945) as follows:

"Mammary tumors do not result from any one of these factors or sets of factors, but from the action and interaction of all three, and it would be folly to attempt to say which is the most important."

Since this theory was advanced and this statement made, it has been observed that the incidence of mammary cancer may be as high in mice where the agent could not be demonstrated by biological assay (Andervont, 1952; Andervont and Dunn, 1948, 1950, 1953; Heston *et al.*, 1950, Heston and Deringer, 1952; Dmochowski, 1953b; and Bittner, 1956) as that found in susceptible breeders with the MTA (Bittner, 1952a and 1953), where all the known causative factors are present.

In 1944 (Heston and Andervont, and Bitter *et al.*) it was demonstrated that the hormonal mechanism associated with the development of mammary cancer in virgin females also was inherited. In these investigations, females of the cancerous A stock, where the virgins have a low but the breeders a high incidence, were crossed with males of the C3H strain, inasmuch as high incidences are found in virgins as well as breeders. The F_1 progeny of this cross had high incidences of mammary cancer, whether they were maintained as breeders or virgins. Woolley *et al.* (1939 and 1941) and Fekete *et al.* (1941) had previously described postcastrational adrenal hyperplasia in animals of some strains and by observing ovariectomized females of the A and C3H strains and their hybrids, a relationship was found between the adrenal changes and a high incidence of mammary cancer in virgin females (Smith and Bittner, 1945, and Smith, 1948). With these preliminary data, other crosses were made between females of the A stock and males of other strains based upon their adrenal response following gonadectomy. The in-

herited hormonal influence which induces mammary cancer was found to be transmitted by several strains, including the I stock, known to be nonsusceptible to spontaneous mammary cancer (Andervont, 1940; Bittner and Huseby, 1950; and Bittner, 1952c). As other studies were completed it became evident that there might be several inherited hormonal patterns, each having a different physiological effect upon the genesis of mammary cancer in mice (Bittner, 1953 and 1954).

In cooperation with Huseby (1948a, 1948b, and 1951), endocrine organs have been transplanted from mice of inbred stocks into experimental F_1 hybrids and the results demonstrate this technique may become a useful tool in studying inherited endocrine differences involving glandular secretion, etc. In one experiment (Huseby and Bittner, 1948a) a difference was found in the hormonal function inherent in the ovaries of two inbred stocks, and in another (Huseby and Bittner, 1951), by transplanting adrenals into adrenalectomized-ovariectomized animals, it was determined that the postcastrational adrenal alterations were controlled by the responsiveness of the adrenal tissue itself.

As may be found in the review articles, the incidence of mammary cancer may be altered by environmental factors, primarily through changes in the normal hormonal stimulation of the glands.

In general, a low incidence of mammary cancer will be observed in any stock lacking any one of the three main causative factors, and so the use of the term "low cancer stock" may have little significance except to those acquainted with the strain, as well as the conditions of the experiments. A stock should *never be called nonsusceptible until breeding females which possess the MTA have been observed*, for the incidence may be no higher in breeders of a susceptible stock lacking the MTA than in nonsusceptible animals. For example, after C57 black (Haagensen and Randall, 1945) and CE (Bittner, 1954 and 1955) animals were nursed by females of cancerous strains, the incidences of mammary tumors in the fostered females and their progeny with the MTA, maintained as breeders, were as high as those reported in some so-called susceptible stocks (see Bittner, 1948), yet in other sublines of the C57 black stock, low incidences have been found in females that possess the agent (Bittner, 1940; and Andervont, 1945a). Although only 4 per cent of the virgin females

of the susceptible A strain develop mammary cancer (Bittner, 1939), when they are crossed with males of the nonsusceptible I stock their AIF_1 progeny, maintained as nonbreeders, showed an incidence of 72 per cent (Bittner, 1952c). This high incidence was due to the introduction of an inherited hormonal pattern by the I stock, which induces mammary cancer in virgin females when they have the inherited susceptibility to mammary cancer and the agent, transferred to the AIF_1 hybrids by their mothers.

Animals of the same line (Andervont, 1945a) of the C57 black stock do not propagate and transfer the "agents" from different sublines of the same cancerous inbred strain with the same effectiveness (Andervont, 1945a; Heston *et al.*, 1945; and Bittner, 1955). Similar data were observed after animals of another nonsusceptible strain had been nursed by females of two inbred cancerous strains (Bittner, 1952c), and it also was found that one female of a litter might possess and transfer the agent while a litter-mate might not. Thus, these conflicting observations could not be explained in accord with any single theory (Bittner, 1955).

Andervont (1952) maintained a colony of wild mice and made the interesting observation that mammary tumors developed in domestic mice which had been nursed by females of the wild stock, showing that the wild mice carried a tumor-agent. However, few tumors appeared in wild mice after they had been fostered by females of the cancerous C3H stock, although the wild mice transferred the MTA for several generations.

Females of either susceptible or resistant stocks may become infected with the agent by the administration (feeding, injection, etc.) of extracts of either normal or cancerous tissues from infected animals, including blood, and by the male at coitus. As soon as the females become infected with the MTA from any source, they will transfer the agent to their progeny in the milk, although the mothers may die noncancerous. The incidence in the injected animals may depend on several factors, including the susceptibility of the test animals, age when injected, activity of the agent, genetic relationship of the test animals to the source material, concentration of the extract, etc.

It is now well established that males of cancerous strains may transfer the MTA and infect agent-free females at coitus (Andervont and Dunn, 1950, 1953; Bittner, 1952b, 1955, 1956, in

press; Bittner and Frantz, 1954; Dmochowski, 1953b; Foulds, 1949; Heston, Deringer, and Dunn, 1956; and Mühlbock, 1952). However, differences have been noted in the sensitivity of females to become infected also, males vary in their ability to infect agent-free females, of either the same or different strains, and this may be altered by foster nursing and the introduction of the MTA of the fostering stock (Bittner, 1952b and 1956).

In one such cross between agent-free C(BALB/c) females and males of the cancer Z(C3H/Bi) stock, 56 per cent of the C females developed mammary cancer and, as indicated by the development of mammary cancer in their CZF_1 progeny, another 25 per cent of the C females died noncancerous but became infected with the agent from the Z males. The number of litters cast by the C females influenced the incidence of mammary cancer, for those which had no more than four litters sired by Z males had a lower incidencce than did those having five and more litters (Bittner, in press).

By observing the CZF_1 offspring born to the C females in successive litters it could be determined that they could become infected at any time between their second and eighth litters. A low incidence (5 per cent) of mammary cancer has been observed in the hybrid (CZF_1) progeny born to noninfected C mothers and a similar incidence was seen for the CZF_1 offspring born to other C mothers before they became infected with the agent from the Z males. After these same females became infected with the MTA from the males, 96 per cent of their offspring developed mammary cancer at an average age of 242 days, and no difference was seen between the young born to infected C mothers whether they died cancerous or noncancerous. Sixty-three young were cast in first litters after their C mothers became infected and they had an average cancer age of 245 days, thus comparing with that recorded for those born to the same mothers in later litters.

The MTA could be demonstrated by biological assay in tumors which developed in the C females and their hybrids following male transmission of the agent, even in tumors which appeared in C females after they were 500 to 600 days of age, and in tumors from the progeny of infected C females which survived to be more than 20 months and died noncancerous (Bittner, 1952, and in press).

The incidence of mammary cancer in C females with amputated uterine horns but with intact ovaries was the same as in unoperated litter-mate controls mated in the same breeding pens with Z males (Bittner and Frantz, 1954). Also, vasectomized Z males may transfer the MTA and infect intact, agent-free C females, and the progeny born to these infected females but sired by agent-free males, will show a high incidence of mammary cancer. The tumors appear in the progeny at an early average cancer age, and the agent could be recovered from tumors from both mothers and progeny (Bittner, in press).

Andervont and Dunn (1948, 1950, and 1953) and Andervont (1950) determined that when males of the C3H/An stock were mated with agent-free C (BALB/c) females, the incidence of mammary cancer in the hybrid progeny might be as high as 60 per cent, and as high for the progeny having either agent-free or agent-carrying fathers. The tumors appeared at an average age in excess of 20 months, and the MTA could be demonstrated only in a few tumors which developed in young animals, as the result of male transmission. These observations have been confirmed in this laboratory (Bittner, 1956). In our study, the average cancer age for the hybrids of the C ♀ × C3H/AnBi ♂ cross was 609 days, and if only the noncancerous breeders are included which survived to 600 days, the incidence of mammary cancer was 51 per cent. In the hybrids observed in our study, the incidence was higher when the animals were born to noncancerous than cancerous C mothers, and was similar for those cast in the first two litters and those born in later litters to the same mothers. Eight tumors from breeding F_1 females, which developed at an average age of 594 days, were tested for the agent but the incidence in the injected females was no higher than in untreated controls (Bittner, 1952b), indicating that the tumors did not possess the agent. Three tumors appeared at an average age of 570 days and no more than one tumor was found in any one assay group.

In our first series of hybrids there was no suggestion of male transmission of the agent yet in a recent experiment a mammary tumor developed in C female, No. 194198, when she was 637 days of age, after having had seven litters sired by a male of the Andervont subline of the cancerous C3H stock. Seven progeny of her first five litters survived to the cancer age; two had tumors

at an average age of 754 days, four died noncancerous at an average age of 660 days, and the seventh is living and free of cancer. Three female hybrids were raised from the seventh litter born to female No. 194198, and all had mammary cancer at an average age of 230 days. The oldest to become cancerous was 288 days of age, and her two mammary tumors were tested for the MTA (the data for the two tumors are combined in the following tabulations at the respective dilutions). Thirteen months following the administration of the extracts of these tumors into ZBC test animals, mammary tumors have developed in 82 of the injected females, an incidence of 67 per cent at an average age of 280 days. Only two of the experimental mice have died noncancerous and 38, or 31 per cent of the total, are alive and under observation. Fifty-seven per cent of those which obtained the material from 2×10^{-2} gm. equiv. of tissue have had cancer compared with an incidence of 79 per cent for those injected with the extracts diluted 1.000 fold; the respective average ages were 289 and 272 days. Thus, when the MTA may be demonstrated in tumors of the C ♀ × C3H/AnBioF$_1$ hybrids, the development of mammary cancer in the test animals does not appear to be dependent upon the concentration of the administered extract. In fact, the MTA extracted from these tumors has proven to be one of the most active to be studied in any assay experiment, regardless of the source material, the stock, or generation of the donor.

Preliminary data indicate that C3H/AnBi males, the descendants of females fostered by females of our cancerous Z(C3H) stock, are "infecting" more C females than did males of the unfostered cancerous C3H/AnBi stock.

Heston and his associates (1950 and 1952) determined that nearly 40 per cent of the breeders of their fostered C3Hb stock would have mammary cancer, compared with only 2 per cent of the virgins (1952); the agent could not be demonstrated when extracts of 24 spontaneous tumors were assayed. The average cancer ages in the agent-free females were in excess of 20 months, while in females of the cancerous line with the MTA the average cancer ages in the virgin and breeding females were 10.6 and 7.8 months, respectively, and the incidences were at least 97 per cent (1950).

In addition to the usual so-called spontaneous tumors, mam-

mary cancer may be induced in agent-free females following the application of methylcholanthrene (Dmochowski and Orr, 1949a and 1949b), and also in males by the addition of estrogen (1949b). The agent could be recovered from the tumors induced in males of the cancerous C3H stock (Dmochowski and Orr, 1949b), but it could not be demonstrated, by biological tests, in the hydrocarbon induced-tumors from agent-free mice (Dmochowski and Orr, 1949b, and Bittner and Kirschbaum, 1950). The former investigators suggested that the action of the carcinogen in the induction of mammary cancer appeared to be independent of the MTA.

By weekly subcutaneous grafting of several hypophyses, Mühlbock (1956) has demonstrated that mammary cancer could be produced in a high percentage of agent-free females of certain inbred stocks and their hybrids and, again, the agent could not be detected in the tumors by biological tests. Mühlbock stated that these tumors must be considered as purely hormonally induced mammary cancers and that when the MTA is present, it acts as an accelerator and intensifier.

The MTA may "appear" suddenly in mice which have neither been nursed by females of cancerous lines nor injected with extracts containing the agent (Bittner, 1941 and 1952b, Dmochowski, 1954). Should this occur in susceptible animals (Bittner, 1941), a high cancerous strain will result and the agent may be demonstrated in the tumors, even from animals of a strain with a low incidence (Dmochowski, 1954).

Based upon the incidences and average cancer ages, the agents from different strains of cancerous animals may not show the same activity when they are studied in the reciprocal F_1 hybrids (see Bittner, 1948), suggesting that these strains may have different "agents."

The MTA of mice has been found to be antigenic and antisera prepared against either normal or cancerous mouse tissues containing the agent have been found to neutralize the agent (Andervont and Bryan, 1944; Bittner and Imagawa, 1955). In some experiments the MTA being assayed had to be obtained from the same source as the antigen used to produce the antiserum in order to obtain neutralization. Dmochowski (1953), in a review of immunological studies on the agent, stated that the data from some studies had to be interpreted on the basis "that

the milk agent is serologically similar to material present in normal tissue of mice."

After comparing the structure of mammary tumors from many groups of inbred mice and their hybrids, both spontaneous and induced, Dunn (1953) reported that while there was greater variation in the histology of tumors where the agent could not be demonstrated, the histology did not reveal the presence of the agent, and Dmochowski (1953b), also observed "a lack of correlation between the microscopic appearance of breast cancer and the presence or absence of the agent."

Summary

Spontaneous mammary cancer in mice generally results from the action of several causative factors, each apparently of equal importance, although there are some exceptions.

The three primary factors are:

The mammary tumor agent (MTA), having the properties of an infectious agent or virus,

The inherited susceptibility for the development of mammary cancer, and,

Hormonal stimulation, without which mammary cancer will not develop.

Several inherited hormonal patterns have been described, one of which induces mammary cancer in virgin females having the other inciting factors, while another hormonal mechanism apparently either inhibits or delays the development of the disease in susceptible breeders with the agent.

There may be genic control of the transmission and propagation of the MTA, end-organ sensitivity, differences in physiologic function and/or response of various endocrine organs to different stimuli, etc. Some may be altered because of environmental factors, including diet and temperature.

While normally the mammary tumor agent of mice is transferred in the milk of infected mothers, it may be administered with extracts of infected tissues, including blood, and by normal and vasectomized males of cancerous lines to infect agent-free females at coitus, even females unable to have progeny with amputated uterine horns.

In mice, the females may become infected by the males at any time between the birth of their second to eighth litters, following

which the mothers will transfer the MTA via the milk. The offspring born to these females prior to infection will show a low incidence. If tumors do occur, they have a late tumor age and usually the agent may not be demonstrated by biological assay. The offspring born to the same mothers following infection, even in the first litters, will have a high incidence, an early cancer age, and extracts of the tumors are biologically active.

Differences have been found in the sensitivity of agent-free females to become infected and of males of various cancerous strains to infect these females, of either the same or of other stocks. This may be altered by foster nursing, or by the introduction of the MTA of another cancerous stock.

Infected mothers will continue to transfer the agent to their offspring, even those sired by agent-free fathers.

The incidence of mammary cancer in susceptible breeding females possessing the agent may be no higher, due to the action of an inhibitory hormonal mechanism, than that observed in another group of breeders where the presence of the MTA in the tumors usually cannot be demonstrated by biological assay.

Purely hormonally-induced mammary tumors have been described in agent-free mice and the theory has been advanced that when the agent is present, it acts as an accelerator and intensifier of tumor production.

It has been suggested that the action of hydrocarbons in the induction of mammary cancer in mice may be independent of the MTA, and while the agent could be recovered from mammary tumors induced in animals of cancerous stocks, it could not be demonstrated in the tumors from agent-free mice.

Antiserum prepared against a specific MTA will neutralize that agent, although an antigenic difference has been reported between the agents from females of a cancerous stock and their progeny of the F_1 generation.

There may be no serological difference between material present in normal tissues of mice and the MTA.

The histology of mammary tumors in mice could not be correlated with the presence or absence of the agent.

If there should be a virus-like agent involved in the development of breast cancer in humans which had properties comparable to the mouse mammary tumor agent, it could be active

in the genesis of some breast cancers but its presence might not be demonstrable in the development of others.

Manifestation of the MTA in mice was dependent upon controlled investigations with cancerous and low cancerous strains and their hybrids. In view of the slow progress being made in the experimental field, especially in obtaining data involving the transmission of the agent, immunological observations, the difficulty in differentiating between agent-containing and tumors where the agent may not be demonstrated without time-consuming biological assay, the sudden appearance of the agent in mice that had neither been fostered nor injected, as well as the difficulty of applying experimental methods used for mice to humans, it would be difficult either to establish or rule out, with our present methods, the role of a comparable agent in the development of the human disease. Due consideration also would have to be given to studies on possible inherited hormonal mechanisms and other genetic factors, including environmental factors, that might influence the interaction of any virus-like agent in the etiology of breast cancer in man.

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HOST VIRUS RELATIONSHIPS IN TUMOR INDUCING VIRUSES

W. Ray Bryan*

The reactions of test animals to tumor viruses are manifested by certain observable qualitative and quantitative phenomena which may be used not only as biological indicators for the assay of virus activity, but also as criteria for characterizing the degree, or relative severity, of the cancerous reaction itself. The use of tumor responses of both the qualitative (*e.g.*, "all-or-none") and quantitative (measurable) types for the purpose of bioassay has recently been discussed in detail (Bryan, 1957). The present discussion deals with the use of the same biological criteria and analytical principles for studies of the cancerous reaction *per se*, particularly in its relations to initiating dose of virus and level of host susceptibility.

It is emphasized that the results of "quantitative" biological experiments directed toward the latter purpose have a considerably lower order of precision than that which is achievable in bioassays of virus activity (Bryan, 1955). This is because the variations among hosts can be eliminated in bioassays, either by statistical methods or through experimental design, and only the controlled average results based upon adequately large numbers of test animals enter into the final calculations of the "analytical" potency determinations. On the other hand, studies on the nature of the cancerous reaction *per se* involve a detailed consideration of the differences that occur in response to a given virus and dose, not only among individual animals of the same genetic type and experimental lot, but also among different experimental lots and genetic types. In addition, it is necessary to compare the analogous neoplastic reactions that occur in various virus-host systems in response to different tumor viruses. As a consequence, the "comparative" relative susceptibility (or reactivity) values inferred from the varied host reactions have a larger experimental error, amounting in some instances to a standard deviation among

* National Cancer Institute, Bethesda, Md., National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

similar individuals of as much as 1 or 2 $\log_{10}$ intervals, or even higher in some tumor virus-host systems. Regardless of this large factor of uncertainty, however, the experimental data have gross quantitative (*i.e.*, order of magnitude) significance since the real differences observed under controlled conditions may extend over a range of as many as 6 or 7 $\log_{10}$ intervals.

Within this frame of reference as to "quantitative" interpretability, certain useful analyses and integrations may be made of available tumor response data which are quantitatively oriented with respect to virus initiating dose or to the amount of virus recoverable from diseased tissues or fluids (*e.g.*, in terms of numbers of virus particles, or of relative doses of a standard virus preparation). Such analyses are presented herewith. Again it is emphasized that the average results and the inter-relationships shown are not presented as precise estimates of the various response parameters, but as gross or "first" approximations which allow certain useful conclusions to be drawn regarding the general characteristics of neoplastic responses to some of the animal tumor viruses.

Results Related To Initiating Dose of Virus

Host Reactions to the Rous Sarcoma Virus

The most widely investigated virus tumor is the Rous sarcoma which was first described as a transplantable neoplasm of the fowl in 1910, by Dr. Peyton Rous of the Rockefeller Institute (1910). One year later he described a filterable agent which was separable from the tumor and which was capable of transmitting the cancerous lesion (Rous, 1911). This agent has been propagated in the laboratory since that time and is now commonly known as the Rous sarcoma virus. Since the largest number and the greatest variety of quantitative investigations have been carried out with this particular agent, data obtained with it are used for an analysis of virus and host factors which influence virus-host interaction, and which therefore determine the nature of this cancerous response.

Inter-relationships among different biological manifestations of the cancerous reaction in the same hosts.

All biological phenomena which have been measured or classified in relation to inducing dose of Rous sarcoma virus, and

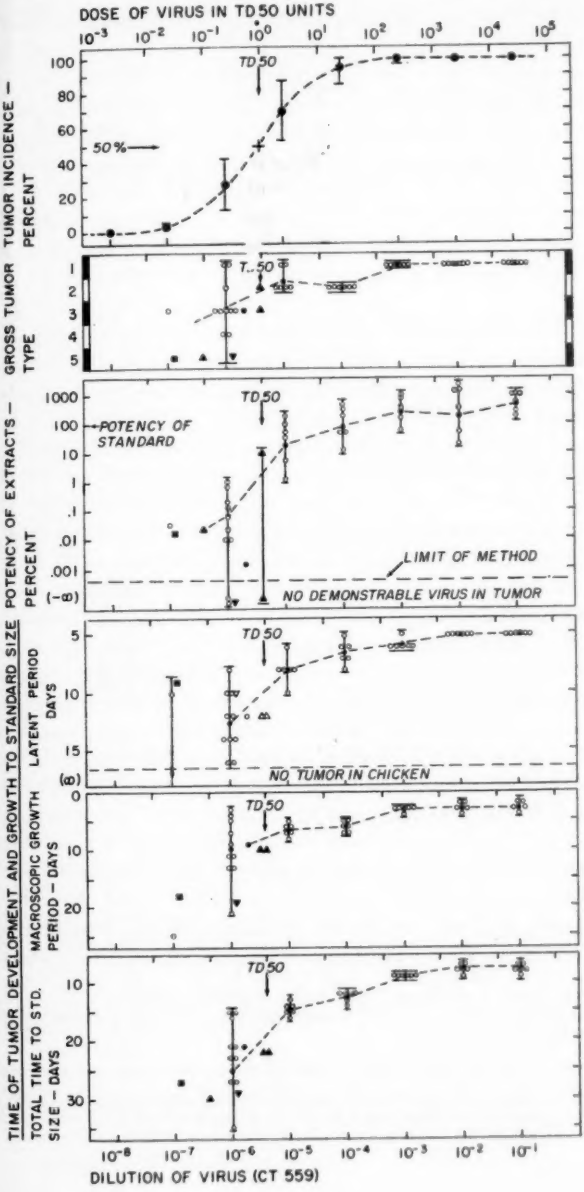
which have been reported to be highly correlated with dose of virus within some significant dose-response range, are included in the category of "biological manifestations" of the cancerous reaction. These include: (1) The incidence of tumors; (2) the latent period, or "tumor induction time"; (3) the rate of growth of tumors: (4) the gross morphological appearance, or "type" of tumor; (5) the amount of virus recoverable from the tumor tissue; (6) the time-to-death of hosts with tumor; and, suggestively, but not yet conclusively, (7) the tendency toward metastasis shown by tumors.

Figure I shows a series of six charts representing as many different manifestations of the cancerous response observed simultaneously in a single large experiment involving a common lot of test chickens and a standard preparation of the Rous sarcoma virus. The detailed data have been previously reported (Bryan, Calnan, and Moloney, 1955) for all but one of the charts.[1] The top chart represents the incidence of tumors[2] observed at successive levels of dilution of the standard virus preparation. The second shows the "gross tumor type" (see footnote 1) of experimental tumors induced at successive dilution levels, of a group which was used for further investigations as detailed

[1] The exception is the second chart, representing "gross tumor type," which was not dealt with in the previous publication. While the experiment was in progress a detailed description was made of each tumor at the time it had reached a certain standard size. The hosts were sacrificed at this particular time and their tumors were taken for potency determinations. For the present purpose, the individual descriptions of tumors were classified and were found to fall into 5 fairly distinct categories. The first 3 categories represented different degrees of involvement with mucoid and hemorrhagic materials, the most involvement being that designated as type 1. Type 4 represented a soft "fleshy" tumor with little or no gross hemorrhagic or mucoid involvement. Type 5 was not observed in the initial experiment (represented by the open circles of Fig. I), but only in supplementary experiments (closed shaped points). Type 5 was characterized by hard slow growing tumors which showed no hemorrhagic or mucoid involvement. Some of them appeared slightly yellow through the skin. Since a particular tumor in a given host may undergo either progression or, more rarely, retrogression at a later time, the types indicated in the chart apply only *at the time of standard size*. They are not intended to represent final diagnoses with respect to degree of malignancy, although, at the particular time represented, they conformed to the different morphological appearances which have been associated by others with the degree of malignancy of this tumor (see Gye and Purdy, 1931).

[2] See: Table 1 of Bryan, Calnan, and Moloney, 1955.

FIG. I. Showing quantitative correlations and variabilities of various biological manifestations of the cancerous reaction in chickens in relation to initiating dose of Rous sarcoma virus.


DOSE OF VIRUS IN TD50 UNITS
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5}
TUMOR INCIDENCE — PERCENT
TD50
50%
GROSS TUMOR TYPE
POTENCY OF EXTRACTS — PERCENT
POTENCY OF STANDARD
LIMIT OF METHOD
NO DEMONSTRABLE VIRUS IN TUMOR
TIME OF TUMOR DEVELOPMENT AND GROWTH TO STANDARD SIZE
LATENT PERIOD DAYS
NO TUMOR IN CHICKEN
MACROSCOPIC GROWTH PERIOD — DAYS
TOTAL TIME TO STD. SIZE — DAYS
10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1}
DILUTION OF VIRUS (CT 559)

in table 3 of the original publication. The remaining charts of figure I represent the respective additional observations which were made on the same tumors.[3] Beginning with the third chart from the top, they are as follows: The amount of virus recoverable from the tumor tissue; the latent period for the appearance of a macroscopic tumor; the time from the first appearance to a standard tumor size (a function[4] of growth rate of the tumor); and, the total time from inoculation of virus to standard size of the tumor (a combination of the two preceding reactions which has been used as a quantitative indicator in bioassays of the virus).

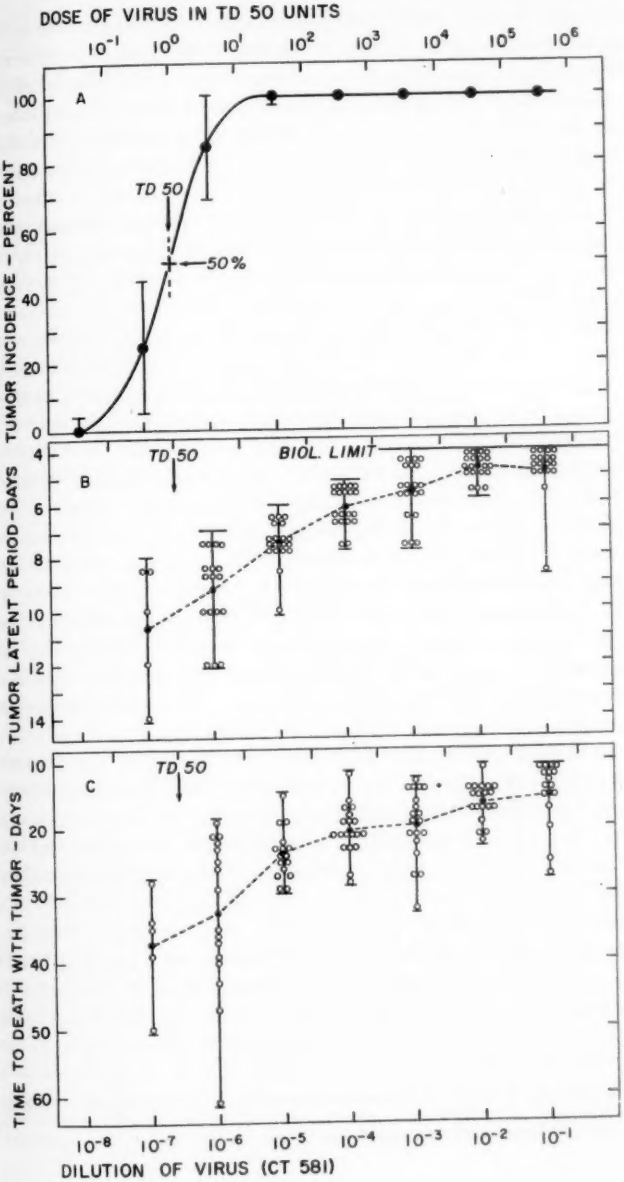
It will be noted that all of the biological criteria approached a maximum (or a minimum in the case of inverse correlations) at the highest virus concentrations (lowest dilutions) and that there were no appreciable differences among individual chickens at these highest doses, except in the amounts of recoverable virus which varied over a range of about 2 $\log_{10}$ intervals. On the other hand, as the dose of virus became progressively weaker all of the criteria showed a significant correlation with dose, as well as an increase in variability among individual animals, within a certain critical dose-response range. In the region of the dose which produced only a 50 per cent incidence of tumors (*i.e.*, TD50), those animals which did develop tumors showed the greatest variability with respect to each of the respective graded criteria. In fact, in this general dose area the individual reactions varied over practically the entire measurable range in each case, some of them being indistinguishable from the uniform reactions to the highest doses, while others were typical of the weakest responses to the lowest doses. The extreme of the weak response in each case was, of course, the negative response, or the failure of some of the individuals to develop any type of detectable reaction at all.

Figure II shows the results of an experiment similar to that above, but in which the tumor bearing chickens were observed

[3] See: Table 3 of Bryan, Calnan, and Moloney, 1955.

[4] The reciprocals of the time values shown are directly proportional to the actual rate constants.

FIG. II. Showing quantitative correlations and variabilities of chicken reactions at successive initiating dose levels of the Rous sarcoma virus: A. Incidence of tumors. B. Tumor latent period. C. Time-to-death with tumor.


DOSE OF VIRUS IN TD 50 UNITS
10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6}
A
TD 50
50%
TUMOR INCIDENCE – PERCENT
0 20 40 60 80 100
B
TD 50
BIOL. LIMIT
TUMOR LATENT PERIOD–DAYS
4 6 8 10 12 14
C
TD 50
TIME TO DEATH WITH TUMOR – DAYS
10 20 30 40 50 60
10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1}
DILUTION OF VIRUS (CT 581)

until death. The latent period and tumor incidence data of the upper two charts have been previously published in detail.[5] The time-to-death data of the lowest chart were not included in the earlier report, which deals primarily with bioassay methods. It will be noted that the death times are inversely correlated with inducing dose of virus in much the same way as the latent periods, although they begin later and spread over much larger intervals of time. As was noted for the other criteria, the variability of the death times increased as the virus dose was decreased, and showed a maximum spread in the region near the 50 per cent dose (TD50).

The time of death of chicks inoculated intracerebrally with the Rous sarcoma virus is also related to dose, even though no macroscopic tumor growths are discernible in the brain tissue at the time of death (Groupé, Rauscher, Levine, and Bryan, 1956; Groupé and Rauscher, 1957*a*). The amounts of virus recoverable from the brains of moribund inoculated chicks are also related to initiating dose (Groupé and Rauscher, 1957*a*).

One of the most important biological manifestations of the malignant neoplasm, and one which is missing from the foregoing group is the tendency toward metastasis (*i.e.*, the incidence and the degree of metastatic involvement among individuals in different dose groups). Although the "metastatic response" has not yet been specifically investigated with respect to virus dose, observations made during the course of other investigations suggest that it also may be related to initiating dose.

In studies on the Rous sarcoma virus in turkeys Groupé and Rauscher (1957*b*) obtained appreciable quantities of extractable virus, and observed a high incidence of metastases, only with those tumors which had been induced with the strongest available doses of standard virus, namely 10^6 ED50. Even at this high dose level the tumors of turkeys were relatively hard and slow growing, although their first appearance was more rapid (*i.e.*, the latent period was shorter) in the turkey poults than in chicks. Other observations indicating a correlation of metastases with initiating dose of virus are those of Duran-Reynals and Freire (1953), and Freire and Duran-Reynals (1953) who found that filtrates from tumors of older chickens (which were weak in "free virus" activity) induced tumors with less metastasizing ca-

[5] See Tables 1 and 6, respectively, of Bryan, 1956.

pacity than filtrates from tumors of young chickens (which were relatively high in "free virus" activity).[6] Similar results relating the degree of activity of filtrates and the tendency of the induced tumors to metastasize were recorded by earlier investigators (Rous and Murphy, 1914; Gye and Purdy, 1931). Although the phenomenon has not been studied quantitatively in this laboratory, it has been observed that highly susceptible chicks dying with tumors as early as 10 to 14 days following inoculations with very strong doses of Rous sarcoma virus, very seldom show metastases, *i.e.*, they die before metastases have had time to show themselves macroscopically. Metastases are frequent, however, at somewhat lower dose levels where the majority of hosts survive for 3 or more weeks. As the above findings indicate, they may be related to initiating dose at these relative dose levels.

A final correlation which should be mentioned in this same connection is that reported by Groupé and collaborators (Groupé, Rauscher, and Bryan, 1956) for the responses of tumor bearing chicks to the biological product xerosin. This substance was capable of delaying the appearance of virus induced Rous sarcomas, and of converting them to a slow growing, atypical, type when the tumors were of "low-dose" origin, but tumors induced with stronger doses of the virus showed no significant differences from those of untreated controls. The initiating dose of virus may thus be a very important consideration when virus induced tumors are used as the test materials in chemotherapeutic studies.

In the instance of all the above phenomena which have been adequately investigated, a similarity is observed in the relationship of the average reaction to the inducing dose of virus, as well as in the degree of variability of the individual chicken reactions about their average at any given dose level. This indicates that all of the reactions are fundamentally related to the initiating dose of virus and that they represent simply different manifestations of the same biological interaction between host and virus. This agent, therefore, appears to be the direct activating cause of this particular neoplasm in all of its manifestations which have been subjected to critical examination in this respect.

[6] These authors interpreted their data in terms of qualitative changes in the virus, *e.g.*, different degrees of "masking," rather than as quantitative differences in inducing dose, or relative virus content of the donor tumors.

A similar conclusion regarding a direct activating role of the Rous sarcoma virus was reached by Rubin (1955) as a result of kinetic studies on the relationship between virus and cells in a tissue culture system.

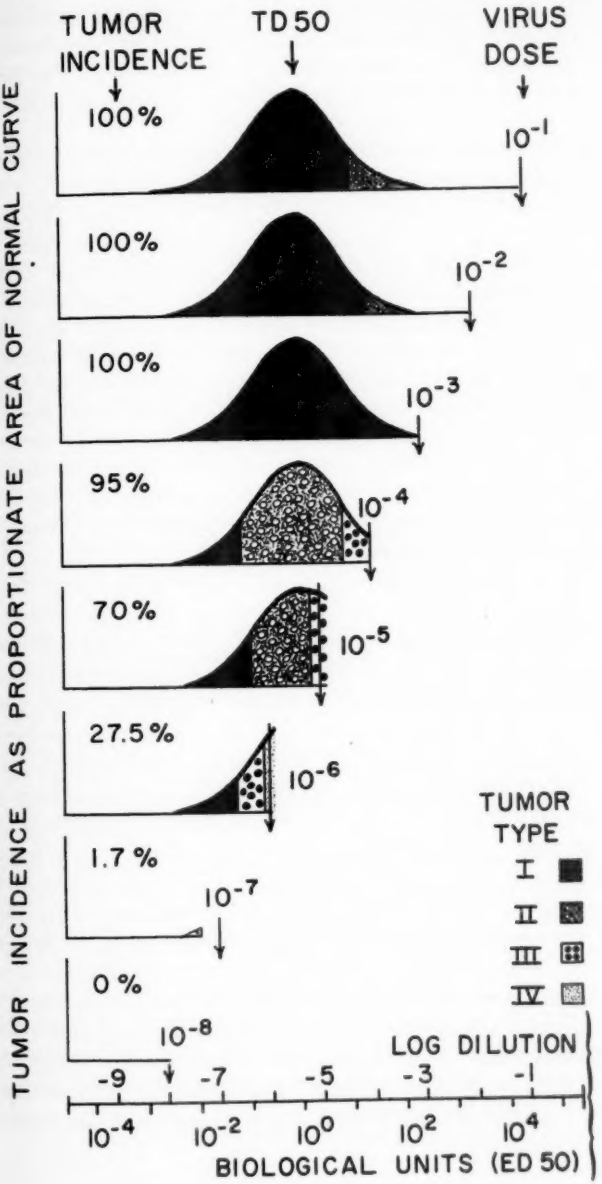
Variations in manifestations (responses) of a given type among different hosts.

Variations among individual chickens of the same experimental lot and genetic type. As was indicated in the preceding section, individual chickens of the same experimental lot and genetic type vary considerably among themselves with respect to specific features of the cancerous reaction. Not only is there variation among chickens of a common group, all members of which have received exactly the same dose, but also the extent of such variation is strikingly related to the magnitude of the initiating dose of virus.

The extent of the over-all variability among individual chickens with respect to the presence or absence of a tumor may be illustrated diagrammatically, using the distribution of their relative "susceptibilities"[7] as inferred from the dose-incidence response curve shown in the top chart of figure I. The bell-shaped curves of figure III represent normal curves having the same standard deviation (in log dose units) as the S shaped curve of figure I (which has the properties of an integrated normal curve —see Bryan, 1957). They may be looked upon as representing the distribution of "just effective" doses, or the doses which would just be required for the production of "positive reactions" in various chickens of the population concerned (see: Gaddum, 1933; Bliss, 1935; and Burn, Finney, and Goodwin, 1950; for discussions of this interpretative procedure). Thus, as indicated by the arrows pointing to the doses administered, any given dose would be expected to produce tumors only in those chickens which were "susceptible" to that or any smaller dose. The areas under the curves up to the arrows represent relative proportions

[7] The analogous term in bioassay literature dealing with responses to toxic compounds would be "tolerances."

FIG. III. Inferred frequency distribution curves for the susceptibilities of chickens to subcutaneous inoculations of a standard preparation of the Rous sarcoma virus (See text).


TUMOR INCIDENCE
TD 50
VIRUS DOSE
100%
10^{-1}
100%
10^{-2}
100%
10^{-3}
95%
10^{-4}
70%
10^{-5}
27.5%
10^{-6}
1.7%
10^{-7}
0%
10^{-8}
TUMOR INCIDENCE AS PROPORTIONATE AREA OF NORMAL CURVE
TUMOR TYPE
I
II
III
IV
LOG DILUTION
-9
-7
-5
-3
-1
10^{-4}
10^{-2}
10^{0}
10^{2}
10^{4}
BIOLOGICAL UNITS (ED 50)

of the treated chickens which would be expected to show a positive reaction, as determined from the fitted dose response curve. The actual points of truncation of the bell-shaped curves correspond to the observed proportions given in per cent along the left border of the figure.

As previously emphasized, the neoplastic response is not an invariable phenomenon which can be dealt with discreetly in the same sharp fashion as, for example, death or survival. Thus, any animal developing a neoplastic lesion of any type or degree of severity would fall into the "positive" quantal category. This does not mean, however, that the lesions would be the same in all individuals, nor that the lesion produced in a highly susceptible individual with a relatively weak dose would be the same as that expected in the same individual with a relatively strong dose of the virus. On the contrary, as illustrated in figure III, the type of lesion produced in a chicken of a given susceptibility is a function of dose. Furthermore, cancerous lesions of a given, less severe type, may result either from the inoculation of weak doses into highly susceptible individuals, or from the inoculation of strong doses into more resistant individuals.

As is evident from figure I, there is a continuous gradation of each of the measurable manifestations of cancer as the dose of virus is decreased, even though the responses to the lowest doses are confined to the most susceptible individuals. Such gradation is illustrated diagrammatically in figure III, using "gross tumor type" for which there were only a few classification categories to be considered. The various patterns of stippling enclose proportions of the total areas under the curves corresponding to observed percentages of individuals showing the different morphological types (all tumors which contributed to the percentage-dose curve of the top chart of figure I were used for this analysis, in contrast to the smaller group used for virus recovery studies, and represented in the second chart of figure I—see also footnote 1).

Variations among chickens of different inbred lines of the same breed. Figure IV shows the responses to a standard preparation of Rous sarcoma virus of two of the inbred lines of White Leghorn chickens (Waters, 1945; 1951) which have been developed at the Regional Poultry Research Laboratory (U. S. Department

of Agriculture) at East Lansing, Michigan. The results[8] were obtained in joint studies with Doctors B. R. Burmester and N. F. Waters of that laboratory. The three charts of figure IV represent different ways of depicting the same group responses in relation to dose (see: Bryan, 1957). It is apparent that the mean susceptibilities of the two lines of chickens, as represented by the respective doses required to give a 50 per cent incidence of tumors (*i.e.*, TD50), were separated by about 4 $\log_{10}$ intervals. The average effective dose was therefore about 10,000 times larger for the more resistant (line 7) than for the more susceptible (line 9) chickens. It will also be noted that both of the dose-response curves were truncated (see: Bryan, 1957), but at opposite ends. As indicated by the lowest chart, this was interpreted to mean that the predominantly highly susceptible line 9 contained a few resistant individuals similar to those of line 7; and, conversely, that the more resistant line 7 contained a few highly susceptible individuals similar to those of line 9. That there could have been a mixture of the lines, experimentally, seems improbable in view of triple checks on the identity of each individual (a separate dye mark for each line, and a numbered wing band and a numbered leg band on each individual chick). It is of interest to point out in this connection that these inbred lines are considered to be only 80 to 90 per cent homogeneous, genetically, at the present time (personal communication).

Variations among fowls of different genera (chickens and turkeys). Groupé and Rauscher (1957*b*) have recently reported comparative responses to a standard preparation of the Rous sarcoma virus obtained in young chicks and turkey poults. The respective dose-tumor incidence curves were practically identical, and well within limits of variation due to experimental error. The latent periods on the other hand were slightly, but significantly shorter in turkey poults than in chicks at a given dose level. Once they had appeared, however, the tumors of turkeys grew more slowly and remained hard in consistency, whereas those of the chickens were soft and grew rapidly.

A very striking difference was found in the amounts of virus recoverable from the turkey tumors as compared with the relative quantities previously reported for chicken tumors at different dose levels, and reproduced in the third chart from the top

[8] To be published jointly with members of that laboratory.

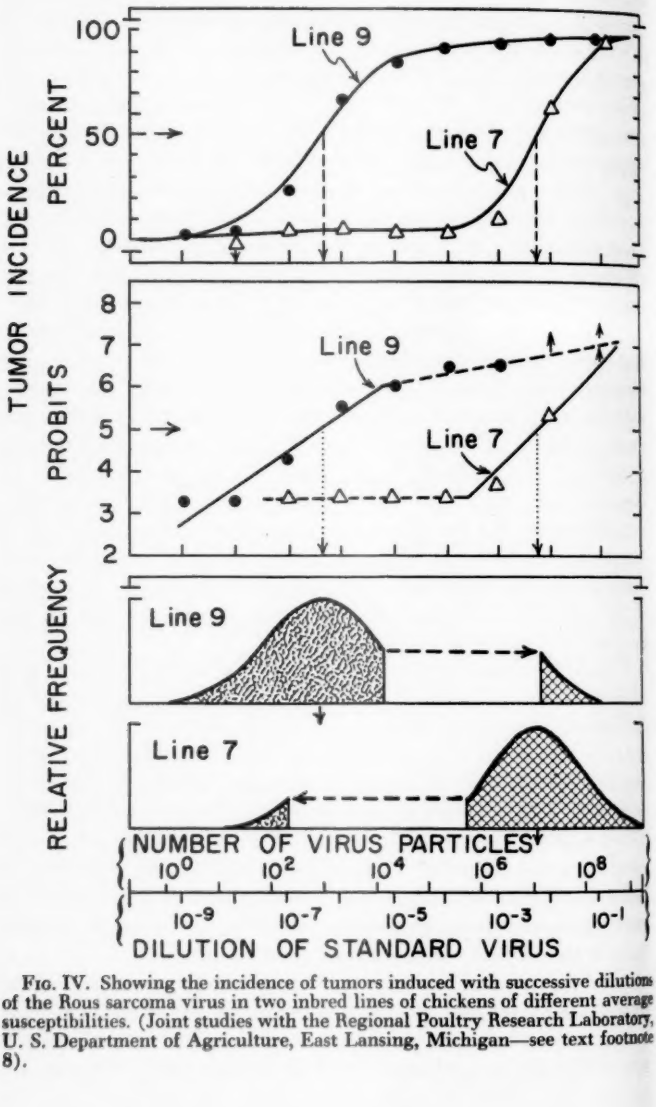


FIG. IV. Showing the incidence of tumors induced with successive dilutions of the Rous sarcoma virus in two inbred lines of chickens of different average susceptibilities. (Joint studies with the Regional Poultry Research Laboratory, U. S. Department of Agriculture, East Lansing, Michigan—see text footnote 8).

of figure I. Using the percentage incidence and latent period results tabled by Groupé and Rauscher for responses of chicks to the standard virus preparation, rough estimates were made of the potencies which correspond to the tabled response values for extracts of their turkey tumors which were tested in chicks. The plot of such estimates is shown in the lower chart of figure V. The upper chart of the same figure shows the incidence of tumors induced in the turkeys by successive 10 fold dilutions of the standard virus. As was pointed out by Groupé and Rauscher, only those tumors induced with the highest dose (containing about 10^6 TD50) yielded appreciable amounts of virus on extraction. The correlation of recoverable virus with inducing dose is therefore shifted to the right (*i.e.*, toward the higher inducing doses) by about 5 $\log_{10}$ intervals, but is otherwise comparable to the results obtained with chicken tumors. The analogy of the turkey tumor findings to those obtained on transfer of Shope papilloma virus from wild to domestic rabbits (*i.e.*, from one genus to another—see Shope, 1933) was emphasized by Groupé and Rauscher.

Host Reactions to Other Tumor Viruses

Beard and co-workers have studied the responses of hosts in relation to dose of the avian myeloblastosis and the Shope papilloma viruses, and in a recent integrative discussion, Beard (1956) has related these results to absolute numbers of virus particles through counts made under the electron microscope. Also, Sharp and Beard (1957) have made similar particle counts on a standard preparation (CT 632) of the Rous sarcoma virus which is employed as a basis of quantitative potency reference in this laboratory. The latter makes possible the orientation of various dose-response studies of this laboratory with respect to numbers of Rous sarcoma virus particles.

Figure VI shows the estimated distributions of "susceptibilities" (see above) of various hosts to these different tumor viruses in relation to numbers of particles contained in the inferred "just effective" doses. The distribution curve at the lower left of the figure represents the Poisson "one particle" distribution which is the theoretical limit under the ideal conditions that: (a) a single virus particle is capable of inducing a detectable neoplastic response; (b) the probability of a particle producing a lesion (*i.e.*,

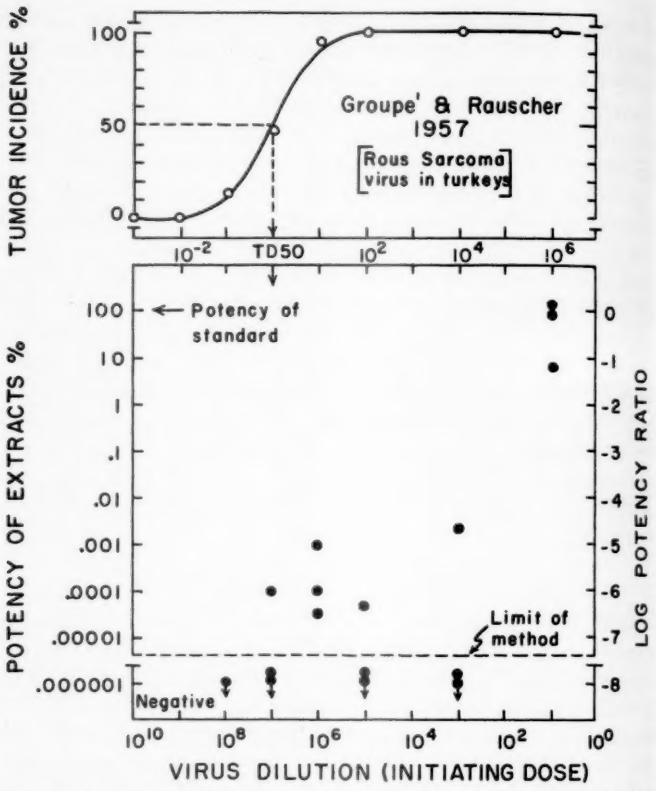


FIG. V. *Upper Chart:* Incidence of tumors in turkey poults induced with successive dilutions of a standard preparation of Rous sarcoma virus. *Lower Chart:* Recovery of virus from tumors taken at random from the group of turkeys represented in the upper chart.
(Prepared from tabled data of Groupé and Rauscher, 1957*b*).

the efficiency ratio) is 1:1; and (c) all hosts are equally and fully susceptible, the only variations in response being due to the chance presence or absence of virus particles in the inoculums.

In terms of the numbers of particles required to produce an effect in 50 per cent of the inoculated hosts, it is seen that the greatest number (80 million) was needed for the induction of papillomas in domestic rabbits by the Shope papilloma virus. About one-third as many (26 million) were required on an aver-

age for the production of myeoblastic leukemia in line 15 chicks, and one-eighth as many (10 million) for the induction of Rous sarcomas in chicks of the most resistant line (7) of the East Lansing White Leghorn strain (see above). On the other hand, New Hampshire Red chicks of the commercial strain employed in this laboratory (as well as White Leghorns of line 9, which had about the same average susceptibility) required only about 600 particles, on an average, for the induction of Rous sarcomas in 50 per cent of the hosts.

One of the most striking facts revealed in figure VI, and one which has been previously emphasized by Beard (1956), is the tremendous spread in the individual susceptibilities of line 15 White Leghorn chicks (as well as of chicks of the New Hampshire Red breed—see Eckert, Beard, and Beard, 1951) to the myeloblastosis virus. Thus, it is seen that the most susceptible individuals would require only a few hundred to a few thousand

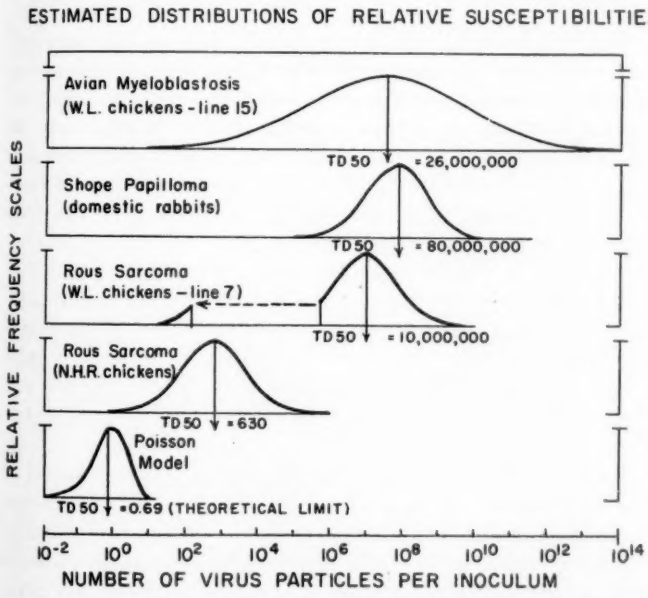


FIG. VI. Comparison of estimated frequency distribution curves for the susceptibilities of specified hosts to various tumor viruses on a basis of the respective numbers of virus particles required to infect.

particles for their infection, whereas the most resistant ones would require over 100 billion particles (Beard gives the 95 per cent and 5 per cent doses as 145 billion and 6,000, respectively, and their ratio as 24 million). The range of variation for the myeloblastosis virus is alone, therefore, about the same as the total range which has been reported for all of the other hosts in response to all of the other tumor viruses. This fact is of the greatest significance to the experimental approach to this tumor-virus problem, and, in the past, made quantitative studies on this agent impractical until methods were developed for (1) the selection of those hosts whose bloods contained the highest titers of virus and (2) the elimination from bioassay groups by truncation analyses of those individuals which showed exceptionally high resistance to the virus (*c.f.*, Eckert, Beard, and Beard, 1954; Beard, Sharp, and Eckert, 1955; Beard, 1957; Bryan, 1957).

Results Related to the Amount of Recoverable Virus

A number of studies on virus tumors have demonstrated quantitative relationships between certain host factors, as well as certain measurable criteria of the neoplastic reaction, and the amount of virus recoverable on extraction of tumor tissues (or cells), as determined by biological assay. One of these, namely that dealing with the low yields of virus obtained with papillomas of domestic rabbits as compared with those obtained with wild rabbit papillomas (Shope, 1933) has already been mentioned. The quantitative differences have been elegantly depicted, diagrammatically, in terms of relative spherical masses of the respective total yields, by Beard (1956). In the case of Rous sarcomas growing in chickens, differences in extractable virus have been shown to be related to the age of the host (Duran-Reynals and Freire (1953); Freire and Duran-Reynals (1953)) and to the age of the tumor itself (Carr, 1943).

Another quantitative relationship of considerable practical as well as fundamental importance is that between the growth rate of tumor cells and the yield of extractable virus per cell, demonstrated by Pontén (1956). The studies involved different lines of Rous sarcoma cells propagated in chickens in the ascites form. Not only was the yield of virus found to be related to the rate at which the cells were growing, but also to the length of time they

had been growing at the particular rate, when the samples were taken for bioassay.

One of the most elucidating quantitative relationships so far demonstrated is that between the quantity of extractable virus and the incidence of tumor cells in which virus particles could be identified under the electron microscope, reported by Epstein (1956). The tumor cells were grown in the ascites form and the virus titers were expressed as multiples of the dose which yielded a 50 per cent incidence of tumors (*i.e.*, TD50). The inset in the lower chart of figure VII shows the data of Epstein's table II relating incidence of cells containing identifiable particles, and virus titer in terms of TD50. The abscissa scale for the entire figure indicates successive 10 fold potency increments above and below a standard virus preparation (CT 632) employed in this laboratory. The upper chart of figure VII shows the average response of test chickens (New Hampshire Red breed) to serial 10 fold dilutions of this particular standard preparation of virus. Correcting for differences in the unit volume of the inoculum, Epstein's results are oriented with those obtained in this laboratory under the assumption that the strains of chickens employed in both laboratories had the same average susceptibility. Even on allowing an error of one log interval for the correctness of this assumption, it is apparent that the weakest cell strain studied by Epstein, which showed a ratio of only 1:3000 cells containing identifiable particles, would still contain a sufficient quantity of extractable virus to induce maximal biological responses in 100 per cent of the test animals employed in this laboratory. Although it is not known that the relationship remains linear when extrapolated beyond the limits studied by Epstein, such extrapolation offers a starting point for judging the magnitude of the experimental undertakings which might be involved in attempts to study the incidence of positive cells at lower and higher levels of extractable virus. For example, the ratio of positive cells to total cells (1:3000) was too low for practical usefulness even in the weakest material encountered by Epstein, and extrapolation of his curve to doses of the present standard virus preparation which would be expected to yield some tumors having no demonstrable virus activity (see Fig. I), indicates that electron microscopy would be entirely impractical for demonstrating the known

fact that such "negative" tumors were actually induced by a virus.

Extrapolation of the curve in the other direction to the level of potency of the standard leads to an expected ratio of about 1:10, whereas extrapolation to 1 $\log_{10}$ interval above the standard gives an expected ratio of about 1:2. If the high correlation observed by Epstein should be found to continue to these higher levels, therefore, the electron microscope might represent a valuable means of detecting tumors of *very high virus content*, as an alternate procedure to biological assays, in the selection of passage materials for the maintenance of highly potent virus-source strains of tumor cells.

The foregoing correlations represent some of those observed

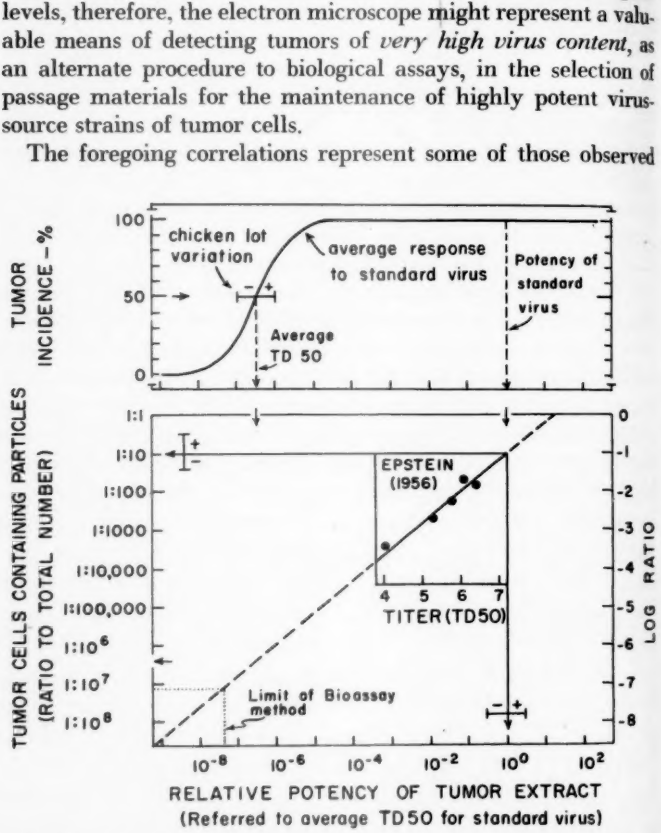


FIG. VII. *Upper Chart:* Average incidence of tumors of chickens in relation to dose of standard Rous sarcoma virus. *Lower Chart:* Incidence of tumor cells in which virus particles could be identified under an electron microscope (Data of Epstein, 1956), referred to dose of standard virus on the assumption of equal chicken susceptibilities (See text).

in relation to the different amounts of Rous sarcoma virus that have been extracted from experimental tumors taken at random, under various specified experimental conditions. As was pointed out in a preceding section, variations in extractable virus are significantly dependent both upon the dose of virus used initially to induce the tumors, and upon the differences that occur among individual hosts in their natural susceptibilities to the virus. Thus, the quantity of virus recoverable on extraction is related to initiating dose on a statistical average basis, at least within certain dose limits. On the other hand, the variation among individual hosts in their responses to a given, weak dose of the virus (*e.g.*, in the region of TD50) may be so great that the tumors of some of them will yield no demonstrable virus at all, whereas others may yield relatively high quantities approaching the levels observed with the strongest initiating doses of virus (see Fig. I). The dependence of recoverable virus upon both the virus and host factors is further illustrated in the following two figures.

The upper chart of figure VIII shows the distribution (in rankits—see Bryan, 1957) of the potencies of extracts of "high-dose"

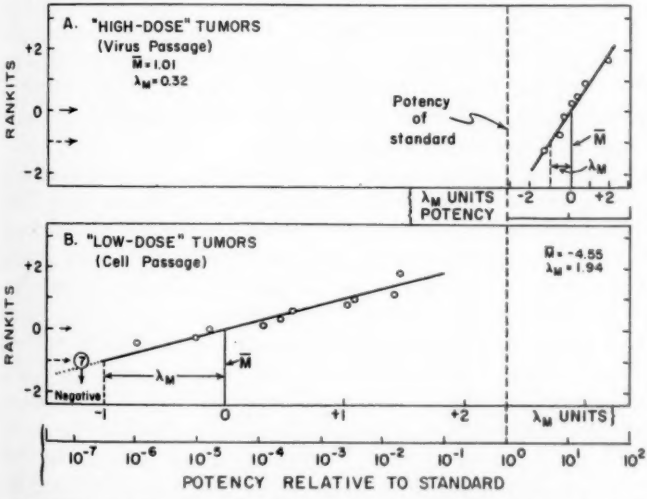


FIG. VIII. Distributions of relative quantities of Rous sarcoma virus recovered: A. In a virus passage series initiated with a "high-dose" of virus. B. In a tumor transplant series initiated with a "low-dose" of virus.

tumors observed during the course of serial passage of the Rous sarcoma virus in chickens. The passage strain was started initially with a strong dose of standard virus, and each successive transfer was made with the extract of the most rapidly developing tumor in a group of 10 chickens inoculated at a given passage. The original potency data for the 16 passages summarized in figure VIII have been previously published (Bryan and Moloney, 1957).

An investigation of a similar nature but involving a "low-dose" tumor and transfer by tumor-tissue transplants rather than by tumor extracts (*i.e.*, virus), has also been carried out in this laboratory[9]. In this instance the original tumor was induced with a very weak dose (dilution 10^{-7}) of a standard virus preparation. At each passage a tumor growing at less than the average rate was selected for continuing the line, but tumors showing unusually slow growth were avoided. A portion of each passage tumor was extracted and a bioassay of the extract was made in relation to a standard virus preparation. The distribution of the potency estimates obtained in 22 successive transplants of the "low-dose" tumor are shown (in rankits) in the lower chart of figure VIII. The normal curves having the same standard deviations (in log dose units) as the rankit curves of figure VIII are shown respectively in the upper and lower charts of figure IX. The latter, bell-shaped, curves represent estimated frequency distributions of the variations about their respective means of the quantities of virus recovered during serial passages of the "high-dose" and "low-dose" tumor lines.

It is seen that extracts of the most rapidly growing tumors of the "high-dose" line contained amounts of virus averaging about 1 $\log_{10}$ interval above the potency of the reference standard, and that the standard deviation (λ_m) of individual potencies about this average was only 0.32 $\log_{10}$ units. The total significant range of variation (*i.e.*, $\pm 2 \times \lambda_m$) was therefore about 1.3 $\log_{10}$ units. In striking contrast, the average quantity of virus extracted from the "low-dose" line of tumors was less than the standard by 4.55 $\log_{10}$ units, and the results for the individual tumors had a standard deviation of 1.94. The total range of variation was in this case about 7.8 $\log_{10}$ units. Twenty-seven per cent of the "low-dose" tumors contained amounts of virus below the limits of sensitivity of the methods employed, and were therefore "negative"

[9] Studies with D. Calnan and J. P. Kvedar, to be published.

for extractable virus. The results of the lower chart illustrate the extreme variations in virus yield which might be expected (and, in fact, were frequently encountered in the past—see: Bryan, 1955) when a transplant tumor line of low average virus content is used as the source of virus for experimental studies. The upper chart, on the other hand, illustrates the high level and the consistency of the virus yields to be expected when rapidly growing tumors of known "high-dose" origin are selected as the source of virus.

General Orientation of Investigative Procedures With Respect to Virus Quantity

In a preceding report to this Symposium Dr. Andervont has presented a table listing the various tumor viruses of laboratory animals, and summarizing them with respect to certain of their biological characteristics. As was emphasized by him, no two of the tumor virus-host systems are exactly alike, but each of them has its own peculiar set of specific host factors, transmittal conditions, and time-response parameters relative to virus-host interaction. It is clear, therefore, that no one system can serve as an

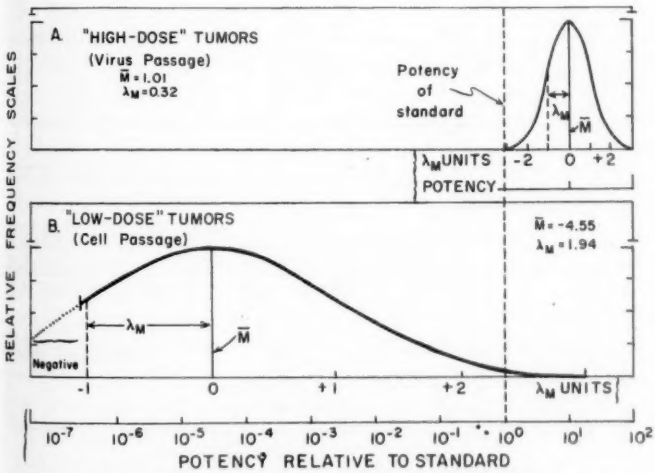


Fig. IX. Estimated frequency distribution curves for the variations in recoverable virus shown in another form in figure VIII (See legend of figure VIII).

exact-type model for the consideration of tumor viruses in general, but that like other host-parasite relationships, the relationships between tumor viruses and their hosts may be expected to exhibit considerable diversity with respect to specific pattern of interaction.

Certain over-all relationships may be expected to be similar for different virus-host systems, however, even though the specific parameters may differ in terms of orders of magnitude. An example of the general orientation of experimental procedures with respect to quantities of virus involved, is presented in figure X. The observed data are those for the Rous sarcoma virus given in preceding sections. They are oriented in the lower chart with respect to numbers of virus particles as well as virus mass, as detailed in the upper chart of the same figure. The curves labeled S and R represent the most susceptible and the most resistant lines, respectively, of chickens that have been encountered in this laboratory. Together they define the limits of virus

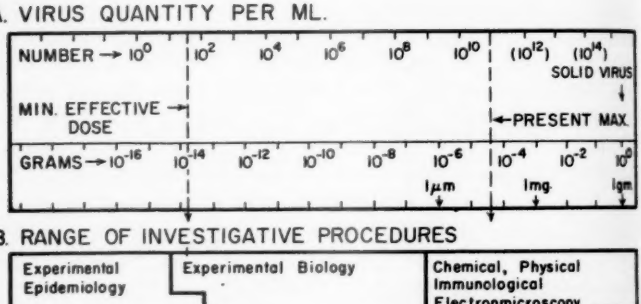


FIG. X. Showing the range of virus quantities involved in laboratory investigations of different types. Illustration based on the Rous sarcoma virus.

dose within which response is correlated with dose, and which must therefore be used for studies on the biological reactions *per se* (*i.e.*, "Experimental Biology"). The broken vertical line at the right represents the upper limit of virus concentration obtainable under the most favorable conditions at the present time. It is just sufficient to permit practical studies under the electron microscope as well as certain chemical and immunological determinations by micro methods. As is indicated, an increase in available quantities of virus by one or two powers of 10 is needed for more definitive chemical, physical, and immunological investigations. Whether the virus would continue to produce neoplasms at such high concentrations, or whether some other reaction (such as the "hemorrhagic disease" observed with present concentrations of this virus when inoculated into late embryos or newly hatched chicks (Duran-Reynals, 1950)) would be obtained, is unknown. The limit of the tumor incidence curve (100 per cent) is therefore extended to this region as a broken line.

At the other extreme of the dose scale a curve (P) is shown for the Poisson distribution of "one-or-more" virus particles in the inoculums (for simplicity of illustration the unit volume of the inoculum is considered to be 1 ml.). This represents the theoretical limit based upon chance variation, of the dose-response curve under the condition that each particle inoculated into a chicken would have a 1:1 chance of inducing a tumor (see above). Although doses of Rous sarcoma virus below that designated as the "minimal effective dose" (broken vertical line at left) do not induce tumors at the sites of inoculation, and are therefore below the range designated as "Experimental Biology," it has not yet been determined whether such small doses might nevertheless result, infrequently, in the induction of tumors at remote sites, after prolonged periods of time (*e.g.*, months or years), as do certain other tumor viruses in other types of hosts. The question of this possibility is raised because in certain instances in this laboratory in which chickens which failed to develop tumors at sites of inoculation, with doses in the region of TD50, were kept for as long as 90 days before being sacrificed, tumors resembling Rous sarcomas were found at remote sites in a few individuals.

It is not improbable, therefore, that doses of Rous sarcoma

virus much smaller than those which yield local tumors at sites of inoculation within a few weeks, may yet be capable of inducing infrequent, but statistically significant numbers of "spontaneous" tumors at remote sites after prolonged periods of time. Because of the numbers of test animals that would be involved in such studies and the fact that they would have to be observed in comparison with adequate controls throughout their natural lives (several years), experiments involving these lower limiting doses of virus would require the techniques of epidemiology. They are therefore designated as "Experimental Epidemiology."

The question may also be raised whether similar variations in type of virus-host interaction may not also occur in other systems under the influence of different combinations of virus quantity and relative level of host susceptibility (*e.g.*, as determined by controllable genetic factors).

Summary and General Comments

Using procedures and principles of analysis of dose-response data similar to those employed for bioassays of virus activity, the complex reactions *per se* of animal hosts to tumor viruses may be analysed and compared on a gross quantitative basis. A quantitative approach to studies on the biology of viral neoplasia is therefore that of considering, individually, the separate measurable or classifiable criteria of the neoplastic reaction, and of comparing them in terms of the respective quantities of virus which are associated with their manifestation (*i.e.*, their "potency equivalents" inferred from their respective dose-response curves).

A summary is given of available quantitative data obtained with various tumor viruses in investigations in which the criteria of biological response have been determined in relation to initiating dose of virus, or to amount of virus recoverable from the neoplastic tissues, or cells. Not only is there an extremely wide variation among the average susceptibilities of different types of hosts to their respective specific tumor viruses, as determined by the numbers of virus particles required to infect, but also in some systems there is a similar wide variation among the susceptibilities of genetically similar individuals to one and the same tumor virus. The virus-tumor problem is therefore characterized by a

diversity of specific patterns of interplay between virus quantity and level of host susceptibility, in determining the nature and outcome of the virus-host interaction leading to neoplasia.

In the instance of one of the tumor viruses which has been most extensively investigated by means of quantitative biological methods, namely the Rous sarcoma virus, and possibly also in the instance of certain other rapidly acting tumor viruses, certain facts and relationships are revealed which are contrary to long held opinions and assumptions regarding the role of tumor viruses in the induction of neoplasia. Thus it is apparent that this particular virus is the direct activating cause of the cancerous reaction which it elicits, in all of its specific manifestations which have been critically investigated in this respect. This is contrary to the belief of many that all tumor viruses, like many other types of cancer provoking agents, act indirectly by "triggering" some other activating mechanism which is inherently present, and potentially activatable, within most living cells of higher organisms.

The second revealment at variance with accepted concepts is that previously reported, but confirmed and extended in studies cited and summarized herein, namely, that the quantity of virus demonstrable in Rous sarcomas is a function of the dose of virus used to initiate them, and, at extremely low initiating dose levels tumors may result which show no demonstrable virus on extraction. This is contrary to the common assumption that tumors having a viral etiology should yield demonstrable virus on extraction, and that failure to do so represents evidence that they are of "nonviral" origin.

Discussion

Dr. Beard: This is undoubtedly some of the most important work that has been done in the field of the tumor viruses. Unless you actually work with these things it's impossible to understand and appreciate the tremendous difficulties that one has to overcome to do any kind of quantitative work with these tumor agents or viruses.

Dr. Dmochowski: Is the average time of tumor appearance that he induces with his present virus a consistent thing, and what is the minimum average time of tumor appearance?

Dr. Bryan: With the strongest doses, the minimum average time is four days. We can see a few small lesions on the fourth day. The inoculation sites are completely negative on the third day. Look at them on the fourth day, and as I pointed out, the tumors will be visible in a few animals. On the fifth day, unless you happen to have some highly resistant animals in your group, one hundred per cent will be positive. It's just that rapid. The higher doses are very precipitate, but as you dilute down to weaker doses the results are highly variable. A highly susceptible animal may show a tumor on the fourth day even with a fairly weak dose. Another one may require thirty days at the same dose. So the individual animal variation is tremendous. But I want to emphasize that you don't get such wide individual variations at the higher doses. The weaker doses are subject to the influence of various other factors that determine the response—hormonal factors I am sure; genetic factors also, since, as we saw differences in genus and strain may greatly alter the response. Many factors are involved when the dose is weak. Dr. Rous has called this high dose material a laboratory monstrosity. Certainly it is not representative of the tumor problem under natural conditions. If we want to study the problem as it is in relation to the cancer problem in nature, then we must go to the weaker doses. We are working at the higher doses and are going backwards in order to be able to predict the requirements for successful experiments with the weaker doses; and I think we are getting to the point where we can do that.

Dr. Pollard, Galveston, Texas: I would like to ask something about the serological response on the part of these chickens to infection with Rous sarcoma. Evidently you have to use a high concentration of virus in order to induce consistent virus-containing tumors. What is the relationship of that to the neutralization phenomena in serums from chickens that are immune to it?

Dr. Bryan: The situation is somewhat the same as the one that Dr. Beard mentioned yesterday. The higher doses kill so quickly that the individuals don't have time to develop much of an antibody response. Those that live longer do develop considerable antibody and if we can take a chicken with a slow-growing tumor—it may be slow for a period of a couple of months, and then

suddenly start growing rapidly and become highly malignant, or it may retrogress, it can go in either direction—if we take the blood from such a chicken that has had a persistent tumor for a long time we usually get a high antibody titer. We are dependent upon chickens with such tumors as our source of anti-serum.

Dr. Stanley: I would like to ask what I suppose is a theoretical question. You can dilute the Rous virus so that you can reach a point where you can get a tumor and no recoverable virus. Now yesterday, Dr. Goodpasture indicated that he could accept as a malignant growth the tumor which metastasizes provided it had no virus. What I would like to know is, when you transplant at the level when you cannot detect virus and you get a tumor and no virus, is that a malignant growth? Or is it a benign growth?

Dr. Bryan: That is a very excellent point, Dr. Stanley. Actually we can by controlling the dose produce classical malignant tumors on an average. We can't tell the individual chickens what to do, and we can't predict what an individual will do, but on an average we can have a large group of classical metastasizing tumors from which you can get no virus. We are dependent on Mother Nature herself for the breaks of the game which permit us to bring this virus back. The one slide that I showed of a tumor that came back rapidly, happened spontaneously. We can watch for the type of individual which shows a rapidly growing tumor and, by transplantation, bring it back to a high virus content, but we cannot change these slow-growing tumors to the more rapidly growing type at will; nevertheless they are classical malignant tumors.

Dr. Beard: I, personally, would like to see this discussion continued for quite a time longer; especially, I would like to get Dr. Goodpasture up here and pin him down this morning.

Dr. Goodpasture: Dr. Stanley has stated that I said that I would accept as a tumor one that showed no virus; that is partially incorrect, because in my paper I tried especially to make it clear, rather that I would not accept as a tumor one that had a virus.

Dr. Beard: We should try to get Dr. Goodpasture out of Houston and somewhere back in a corner so about four or five of us could

work him over. You know he gets us here one at a time and by virtue of the weight of his information and past experience he can sort of beat our ears down. But, if we could get about four or five people on him at one time, I think we could addle him by making him admit something.

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ELECTRON MICROSCOPY OF TUMORS OF KNOWN AND SUSPECTED VIRAL ETIOLOGY

LEON DMOCHOWSKI AND CLIFFORD E. GREY*

Viruses are one of the factors involved in the origin of a number of tumors and are suspected of playing a part in the development of at least some other tumors (for review of literature, see Dmochowski, 1956a). An intimate knowledge of the tumor-inducing viruses may lead to a better understanding of how to deal with these agents and thus perhaps lead to the prevention of some tumors of known viral origin. A proper understanding of the viruses involved in the origin of these tumors may in turn serve as a basis of a search for similar agents in those types of human cancer in which a virus may conceivably be one of the responsible factors.

One of the ways to study any of the disease-inducing agents is to study these agents in the specific lesions they induce. It appeared, therefore, that a study of tumor-inducing viruses in tumors which they induce might lead to a better understanding of these agents. Electron microscopy of ultra-thin sections of virus-induced tumors prepared in the usual way for electron microscopy was selected as a method of study of tumor-inducing viruses. An RCA EMU-3A electron microscope was used in the present study.

Electron microscopy during the past few years has provided a better understanding of normal cell constituents, has led to the discovery of new elements previously unknown and has clarified our knowledge of other constituents either controversial or only suspected of their existence. Further, it has also led to our understanding of the structure of ordinary viruses in tissues they infect (Dmochowski, 1956a). These viruses have been found to possess a characteristic structure. Although this structure is

* Section of Virology and Electron Microscopy, The University of Texas M. D. Anderson Hospital and Tumor Institute and Department of Microbiology, Baylor University College of Medicine, Texas Medical Center, Houston, Texas. These investigations were supported by U.S.P.H.S. Research Grants No. C-2612 and C-2952 from the National Cancer Institute, National Institutes of Health, Public Health Service and by American Cancer Society Grant-in-Aid No. VI-10A.

complicated and at the present moment not yet understood, it helps in differentiating viruses from normal cell constituents.

The required knowledge and background were therefore available as a starting point for an electron microscope study of tumors of either known or suspected viral etiology and for a study of the agents involved in their origin.

A recently completed long-term study of mammary tumors of mice will be briefly presented as it has already been reported elsewhere on several occasions (Dmochowski, Haagensen, and Moore, 1954, 1955; Dmochowski, 1956b; Dmochowski and Grey, 1956). The original results of this study have since been confirmed by a number of workers (Bernhard, Bauer, Guérin, and Oberling, 1955; Bang, Vellisto, and Libert, 1956; Bang, Andervont, and Vellisto, 1956).

Characteristic virus-like particles have been found in most of the mammary tumors of virus-carrying strains of mice and in a number of breast tumors of apparently virus-free mice. Biological tests, however, failed to give conclusive evidence that this is the specific virus, one of the factors responsible for the origin of mammary tumors in mice (Dmochowski, 1956b). Known cytoplasmic elements of cells such as mitochondria have frequently been found to lose their characteristic structure, ergastoplasm or endoplasmic reticulum to form large vacuoles or vesicles and the microsomes or submicroscopic particles to disappear, in cells of mammary tumors of mice.

The characteristic structure of virus-like particles found in mammary tumors of mice may be seen in figure I. It shows a small duct (D) with virus-like particles present in the duct, some lying free in the duct, others along the cell membranes (CM) or near cytoplasmic processes (CP). Mitochondria (M) in various stages of degeneration are seen in the cytoplasm of one of the tumor cells, with aggregations of the cytoplasm (CY) in which inclusion bodies have been observed to form and termed viroplasm. The ergastoplasm (ER) appears unchanged.

Similar picture may be seen in the section of another high-cancer-strain mammary tumor, shown in figure 2. Cytoplasmic processes (CP) with some virus-like particles (VP) lying in the duct are seen. However, an almost identical picture is shown in figure 3 which shows the cytoplasm (CY) of a tumor of an apparently virus-free-strain mouse with vesicular-type (VESP)

particles. Outside the cellular membrane (CM) of the cell in the intercellular space are seen characteristic virus-like particles (VP) with cytoplasmic processes (CP) extending into the space. An even greater concentration of virus-like particles (VP) may be seen in another apparently virus-free-strain mammary tumor, shown in figure 4. Virus-like particles are seen lying in the intercellular space (IS), with cytoplasmic processes (CP) extending into space. It may well be that the virus-like particles are extruded through the cytoplasmic processes. In the cytoplasm (CY) of a tumor cell an aggregation of vesicular-type particles (VESP) may be seen. It is unknown whether the vesicular-type particles are a stage in the formation of the characteristic virus-like particles with an internal dense zone or are an expression of the reaction of the cell to the presence of the virus-like particles, varying in size from 700Å to 1450Å.

This study has shown that there exist in a tumor of undoubted viral origin particles which closely resemble particles of ordinary viruses encountered in specific lesions by a number of workers (Dmochowski, 1956a). Further, there exists a close similarity between the particles observed in mammary tumors of mice and those encountered in other tumors as observed in the studies which will be presented.

It is known that mice carry a number of latent viruses, such as pneumonia virus of mice, lymphocytic choriomeningitis, Newcastle disease, Kilham virus, and other viruses (Dmochowski, 1956a). It is unlikely that the mentioned agents are those found in the electron microscope studies of mammary tumors of mice, although the relationship with the Kilham virus (Kilham and Murphy, 1953) may require further elucidation.

The problem of viral origin of mouse leukemia has been the subject of extensive studies carried out by Gross (1950, 1953, 1956) and recently confirmed by G. W. Woolley and Small (1956) and in some aspects by L. W. Law, S. E. Stewart, A. D. Dulaney, G. Rudali, J. F. Duplan, and R. Latarjet (Dmochowski and Grey, 1956). In collaboration with Dr. L. W. Law of the National Cancer Institute, Bethesda, Maryland, studies were therefore undertaken on organs of mice of two strains (AKR and C58) with a high incidence of spontaneous lymphatic leukemia. Young, 6–8 weeks-old mice of both strains were used as controls. Preliminary results of this study have already been described

(Dmochowski, Grey, and Law, 1956) and further analyzed in a subsequent paper (Dmochowski and Grey, 1956).

These studies will be briefly presented in an attempt to show some essential similarities in the morphological structure of leukemic mouse cells and chicken lymphomatosis cells, and some differences between the morphology of these tumors and that of mouse mammary tumors.

Organs of young control mice have so far failed to reveal any virus-like particles or any other changes observed in organs of mice with spontaneous leukemia. In the thymus gland, spleen, cervical, mesenteric and other lymph nodes of mice of the AKR and C58 strains, in advanced stages of leukemia, virus-like particles have been observed as well as a number of characteristic changes in the cells. These changes appeared to follow the same pattern and consisted of various stages of cellular breakdown. It may be that with the development of virus-like particles, appearance of inclusion bodies, formation of virus-like particles in these bodies, a progressive damage occurs in the cytoplasm of cells. Progressive destruction of cell elements, gradual vacuolization, formation of large unidentified bodies may take place simultaneously or as a result of the appearance of virus-like particles. The release of virus-like particles appears to follow complete disintegration of the cells. The number of virus-like particles observed may vary from a few scattered inside or along the edge of the cytoplasm of cells to a considerable number found in the intercellular spaces.

The appearance of normal cells of a cervical lymph node of an AKR strain mouse is shown in figure 5. The nuclei (N) nucleoli (NU) appear well preserved; the cytoplasm (CY) shows distinct cellular membranes and normal-looking mitochondria (M). In many respects normal appearing cells of a cervical lymph node of a mouse with spontaneous leukemia are seen in figure 6. The nuclei of cells (N) appear normal. Some mitochondria (M) appear unchanged and others show various stages of degeneration. In the cytoplasm (CY) of some cells formation of virus-like particles (VP) and inclusion bodies (IB) takes place. Some normal cells, some affected cells and remnants of broken-down cells are shown in figure 7. Inclusion bodies (IB) in the initial stages of formation may be seen. Cellular fragments (CF) with a group of virus-like particles (VP) may also be ob-

served. The characteristic structure of virus particles lying in an intercellular space is shown in figure 8. They show a dense nucleoid structure either centrally or excentrically placed and a distinct membrane surrounding a lighter part of the particle. The size of virus-like particles encountered in leukemic organs or tissues of AKR and C58 strain mice has been found to vary from 900Å–1650Å and the size of the nucleoid structure from 250Å to 700Å.

No particles, resembling virus particles have been observed in normal organs of mice of the same strains. It is of course possible that they may be present in considerably smaller numbers, in these organs. Additional studies are now being carried out to verify this point.

In collaboration with Dr. L. Gross of the Veterans Administration Hospital, Bronx, New York, a study was undertaken of lymphatic leukemia induced by cell-free extracts of organs of leukemic mice. In this study organs of C3H strain mice with induced leukemia have been examined. Similar changes to those seen in spontaneous leukemia have been observed in the cells of induced leukemia. They appear to follow a similar pattern and in addition virus-like particles have been observed in the sections of leukemic organs, such as thymus and lymph nodes.

Fragments of a broken-down cell surrounded by apparently normal cells, showing nuclei (N), cytoplasm (CY) and mitochondria (M) are seen in figure 9. In addition, a large osmiophilic body, oval-shaped, may be seen among the cell fragments. These bodies have been frequently encountered in both spontaneous and induced leukemia of mice and their nature and connection with leukemia requires investigation. Varying size, densely osmiophilic bodies (LB), termed lipid bodies, have also been encountered frequently in both types of leukemia, and are shown in figure 10. They are seen lying in the cytoplasm of cells undergoing degeneration, as can be seen in mitochondria in various stages of break-down, vacuoles of varying sizes and in the absence of cytoplasmic granulation. For comparison, apparently normal cells with nuclei (N), cytoplasm (CY) and mitochondria (M) are shown in the upper part of the picture.

Characteristic virus-like particles (VP) showing internal structure are seen in the intercellular space, as shown in figure 11. The space is surrounded by mostly normal looking cells show-

ing nuclei (N), mitochondria (M) with their characteristic structure and finely granular cytoplasm (CY). In one of the cells, Golgi apparatus (GA) is seen near a lipid body (LB). Virus-like particles (VP) in large numbers, lying in what appears to be fragment of a degenerated cell or cells, are seen in figure 12. Among the fragments of cells and virus-like particles a degenerating mitochondrion (M) is seen. Part of two cells are also seen; one apparently normal and another with degenerating mitochondria and with an unknown body (UB).

An estimation of the size of virus-like particles in induced leukemia has shown the size to vary from 940Å to 1,800Å with the nucleoid structure varying from 300Å to 780Å. The size of these particles therefore falls within the size range of particles observed in spontaneous leukemia.

As in the case of mammary cancer in mice, it is realized that these virus-like particles may be latent viruses, unconnected with leukemia itself. They may be present in leukemic organs of mice by virtue of a possible attraction by diseased cells which may tend to localize a latent or symbiont agent. Further studies are in progress to establish what relationship, if any, exists between the virus-like particles encountered in the two types of leukemia and between these particles and the origin of leukemia.

A most striking difference between the appearance of cells of mammary tumors of mice and that of cells in leukemia in mice was found to be based on the frequently encountered cellular break-down in the case of the latter. When the relationship between the virus-like particles present in sections of leukemic organs of mice and leukemia is established, it may then be possible to establish the real character of these changes in the appearance of leukemic cells.

A preliminary study of lymphatic leukemia induced in C3H strain mice by x-ray irradiation carried out in collaboration with Dr. L. Gross has, so far, failed to reveal virus-like particles in the leukemic organs of these mice. It may be that here again as in normal organs of young mice of AKR and C58 strains, the virus-like particles are present but in small numbers difficult to encounter in sections constituting only a very small part of a cell. These results, if confirmed, may indicate that there may be at least two different types of leukemia in mice.

The studies on various types of leukemia are being continued.

Preliminary observations on granulocytic leukemia of mice, maintained in an ascites form as a transplantable tumor may be mentioned. These studies are being carried out in collaboration with Dr. L. W. Law of the National Cancer Institute, Bethesda, Maryland. Virus-like particles have been observed in the cells of this type of leukemia. They are, however, found in considerably smaller numbers, usually intracellularly and no signs of cellular breakdown have been observed. They appear to be closely related to ergastoplasm. The appearence of cells of granulocytic leukemia may be seen in figure 13. The cells show nuclei (N) and in their cytoplasm mitochondria (M), isolated virus-like particles (VP) and lipid body (LB) is seen. A single cell at higher magnification is shown in figure 14. The nucleus (N), nuclear membrane (NM), finely granular cytoplasm (CY) with mitochondria (M) are shown. In addition, virus-like particles are present.

So far, no virus-like particles similar to those found in spontaneous and induced leukemia have been encountered in the transplantable form of granulocytic leukemia. The virus-like particles observed in cells of granulocytic leukemia resemble the spherical particles inside the ergastoplasm (endoplasmic reticulum) of Ehrlich ascites tumor cells described by several authors (Friedlaender, Moore, Love, Brown, and Koprowski, 1955; Friedlaender, Moore, and Koprowski, 1955; Friedlaender and Moore, 1956). These dense, spherical particles, measuring 600Å in diameter were originally associated with one of the viruses used for experimental infection of Ehrlich ascites tumor cells and were later also observed in uninoculated cells of similar origin. Similar particles have also been observed in Ehrlich ascites tumor cells by other workers (Adams and Prince, 1956). Particles of similar appearance have also been observed in spontaneous mammary tumors of mice (Dmochowski, 1956a,b; Bernhard, Bauer, Guérin, and Oberling, 1955). The size of virus-like particles in cells of granulocytic leukemia of mice has been found to vary from 450Å to 600Å, and therefore falls within the size range of virus-like particles observed in Ehrlich ascites cells, normal or infected with Anopheles A virus, or in mammary tumor cells of mice. It is not known whether they are a normal, hitherto unknown cell component, an expression of cell-defence by the ergastoplasm to virus infection or a stage in the develop-

ment of virus particles. Further studies are certainly required to elucidate this problem and are under way.

Lymphomatosis in chickens is a frequently encountered neoplasia of chickens, known to be caused by a viral agent with typical properties of an infectious agent (for literature see Dmochowski, 1956a). In collaboration with Dr. B. R. Burmester of the Regional Poultry Research Laboratory of the Agricultural Research Service, U.S. Dept. of Agriculture, East Lansing, Mich., a study of some types of this tumor, especially visceral lymphomatosis, has been initiated. The strain of visceral lymphomatosis, used in the present study, was the RPL–12 lymphoid tumor, which originated as a field case of visceral lymphomatosis (Olson, 1951). A filterable agent in this tumor was found to induce tumors not at the site of inoculation but in the viscera of chickens after an interval of time varying from 1–6 months. Organs of chickens affected by visceral lymphomatosis were therefore examined for the presence of characteristic changes which could be correlated with the presence of a virus.

In the present study, organs such as liver and spleen were used from chickens with lymphomatosis induced by intravenous inoculation of chickens at the age of 1 day to 3 weeks with cell-free filtrates of the sixteenth to seventeenth passage of strain RPL–12 tumor. The organs from birds showing extensive tumor involvement were examined in a number of cases. Preliminary results of this study have already been published (Dmochowski, Grey, and Burmester, 1957).

The cytoplasm of cells of the spleen and liver showed a progressive destruction of cell elements such as mitochondria and ergastoplasm. Large osmiophilic bodies were found in the cytoplasm of the cells. Formation of virus-like particles takes place within these bodies and following break-down of cells, the particles are released into the surrounding intercellular spaces. The virus-like particles, according to preliminary estimation based on a comparatively small number of particles, vary in size from 700Å to 900Å and their internal dense core from 280Å to 350Å. In similar organs of normal control chickens of the same strain similar changes have, so far, not been observed.

The appearance of cells from an unaffected part of liver is shown in figure 15. Finely granular cytoplasm (CY) with mitochondria (M) can be seen. In addition cytoplasmic processes

(CP) extending into a small billiary duct are shown. They have recently been described in greater detail in the rat liver (Rouiller, 1956). Cells of a liver showing some of the changes frequently encountered are seen in figure 16. Cytoplasm (CY) shows loss of fine granular structure; mitochondria (M) show signs of degeneration. Around the mitochondria ergastoplasm (ER) may be seen; cytoplasmic processes are clearly shown and a number of lipid bodies (LB) are present. A more advanced stage in liver cell damage is seen in figure 17. In the cytoplasm (CY) of cells, inclusion bodies (IB) are present in an advanced stage of formation. The cytoplasm shows condensation and loss of fine granular structure. A comparison between an almost normal-looking cell of lymphomatosis liver and a cell in advanced stage of damage is seen in figure 18. In the former cell, nucleus (N) with nucleoli (NU) is seen with the cytoplasm showing mitochondria and some vacuoles. In the damaged cell, vacuoles (V) are present with inclusion bodies showing the presence of virus-like particles (VP).

The appearance of the part of two cells in lymphomatosis spleen is shown in figure 19. The cells separated by cellular membranes (CM) show nucleus (N), an unknown body (UB) and well preserved mitochondria (M). Inclusion bodies (IB) with virus-like particles (VP) within these bodies and some lying freely in the cytoplasm are seen in another section of the affected spleen (Fig. 20). Vacuolization in the cytoplasm of cells in the lymphomatous spleen, loss of granular structure of the cytoplasm of cells, with some mitochondria (M) showing early signs of damage are seen in figure 21. Some ergastoplasm (ER) is present and cellular membranes (CM) are in part preserved. An osmiophilic body, described as unknown body (UB) is present and also an inclusion body (IB) showing virus particles (VP). Virus-like particles present between the cell membranes (CM) of two adjoining cells of spleen are seen in figure 22. In one of the cells mitochondria are pointed out, with one structure showing virus particles, which may have been a mitochondrion which has undergone vacuolization. Virus-like particles at higher magnification, lying near a cytoplasmic process are shown in figure 23.

It is, as yet, unknown whether the virus-like particles found in organs of chickens affected by visceral lymphomatosis are the

specific virus. Experiments are now being carried out to elucidate the nature of these particles and their relationship to visceral lymphomatosis.

Following the observation of virus-like particles in Rous sarcoma cells grown *in vitro* (Claude, Porter, and Pickles, 1947), repeated attempts to confirm this observation on Rous sarcoma cells grown in tissue culture met with no success (Bernhard and Oberling, 1953) or with only partial success (Bernhard, Dontcheff, Oberling, and Vigier, 1953; Oberling, Bernhard, Dontcheff, and Vigier, 1954). The presence of spherical particles, occurring either in pairs or in small clusters in sections of two Rous sarcoma tumors, mostly on the surface of tumor cells and occasionally in the cytoplasm was reported by Gaylord (1955) and also in small numbers by Bernhard (1956). The diameter of virus-like particles in these studies varied between 670Å to 870Å (Claude *et al.*, 1947) to approximately 500Å (Gaylord, 1955.) It was difficult to decide whether the virus-like particles multiply inside or outside the cells and their relationship to Rous virus was not established in this study.

Experiments were therefore started on Rous sarcoma tumors induced with a strong, standardized Rous virus preparation by Dr. W. Ray Bryan of the National Cancer Institute, Bethesda, Maryland. The Rous tumors were obtained from chickens inoculated by Dr. Bryan with the 27th serial virus passage in chickens of a line started two years back and described as CT 629 standard virus preparation (Moloney, 1956).

In a series of six chickens inoculated with the potent standard virus preparations, all six developed tumors, of which two so far have been examined. One of the tumors was examined on the second day and the other tumor a week following their appearance. The second tumor was twice the size of the first tumor.

Many virus-like particles similar to those reported previously (Gaylord, 1955) have been observed in the Rous sarcoma tumor taken for study on the second day of its appearance. The particles appear spherical, uniform, consisting of a dense central mass surrounded by what appears to be an outer limiting membrane. Particles have been found either lying singly or in small groups usually on the outer surface of cell membranes or in vacuoles of the cell cytoplasm. Some cellular changes have been observed, such as various stages of the loss of internal structure of mito-

chondria, some swelling of the ergastoplasm, and vacuolization of the cytoplasm of tumor cells. Lipid bodies have also been observed in the cytoplasm. The particles have been found to vary from 700Å to 950Å with the internal dense zone varying in diameter from 300Å to 400Å. They appeared therefore to be somewhat larger than those observed by Gaylord (1955) which varied from 450Å to 600Å.

The appearance of ergastoplasm (ER) in a Rous sarcoma tumor cell and of a mitochondrion (M) is shown in figure 24. In addition, a dense osmiophilic body is present. A cluster of virus-like particles (VP) lying outside the cell membrane is seen in figure 25. It also shows nucleus (N) with a nucleolus (NU) and cytoplasm (CY) of cells with ergastoplasm (ER). Virus-like particles lying singly, in pairs and in a cluster outside a cell membrane (CM) are seen in figure 26. The cytoplasm (CY) of cells shows ergastoplasm (ER) and some mitochondria (M).

No virus-like particles have been observed in cells of the Rous sarcoma tumor taken for examination a week following its appearance. It is doubtful if the difference between the results of the study of the two tumors is based on the older age of the second tumor. According to Dr. W. Ray Bryan, the potency of the extract injected into the chickens sent for electron microscope study was found to be ten times higher than the potency of the standard (CT 629). Dr. Bryan states that what an individual chicken can do to the virus is extremely variable. The variation in recoverable virus among different chickens inoculated with exactly the same dose is over 2 logs, assuming no unusually resistant chickens are found in the inoculated series. If, according to Dr. Bryan, one of the latter chickens gets into the group, the recoverable virus may be dropped many log intervals, and one would see no particles in the tumors of such animals.

The remaining four tumors of the inoculated chickens, taken at different intervals of time following their appearances, are now being examined and it will be interesting to see what further variations are found in the content of virus particles in these tumors.

The virus-like particles found in the Rous sarcoma tumor may be the agent itself although additional experiments are planned to investigate this problem. It may be of interest that the size of virus-like particles found in the Rous sarcoma tumor falls within

the size-range of virus-like particles found in chicken lymphomatosis. It offers an opportunity for a speculation on the relationship between the two viral agents, assuming the particles are the respective specific agents.

In view of the observations on mouse leukemia and chicken lymphomatosis, preliminary studies have been carried out on human leukemia in collaboration with Dr. C. C. Shullenberger and C. D. Howe of the Department of Medicine of the M. D. Anderson Hospital and Tumor Institute. It appeared advisable to study first cases of human acute leukemia, preferably untreated and to use as a source of material not blood of the patients but biopsy specimens of the affected organs.

So far, one case of acute lymphatic leukemia of a 15 year-old girl was studied and biopsy material from an enlarged cervical lymph node used. In cells of the affected lymph node a number of changes have been observed which are comparable with those observed in cells of the affected organs in mouse leukemia and chicken lymphomatosis. Vacuolization of the cytoplasm of cells, breakdown of mitochondria, swelling of the ergastoplasm, formation of large unidentified bodies and of inclusion-like bodies have all been observed. In addition, what appear to be virus-like particles, approximately 1000Å in diameter have been observed in the intercellular spaces and within inclusion-bodies. The significance of these preliminary observations can only be assessed by further extensive studies which are now being carried out.

The appearance of blood vessels observed in the affected cervical lymph node and of the surrounding tissues is shown in a longitudinal section in figure 27 and in a transverse section in figure 28. The capillary wall can be distinctly seen, with the double base membrane of the capillary wall in figure 27. In the lumen of the blood capillary in both sections a white blood corpuscle is present. Some of the changes observed are shown in figure 29. Vacuolization (V) of the cytoplasm (CY) with the formation of large osmiophilic bodies (UB) and normal looking nuclei (N) are present. Large size unknown body (UB) with mitochondria in various stages of degeneration is shown in figure 30. A large number of virus-like particles (VP) in an intercellular space may be seen in figure 31. The particles at higher magnification are seen in figure 32. In one place they are seen in a group without a visible surrounding membrane, in another place

(IB) part of a membrane surrounding the group of particles may be seen. It is difficult at the present time to form an opinion whether the formation of inclusion bodies is unconnected with mitochondria or whether it takes place within them as the mitochondria lose their structure and become filled with a homogenous substance. A large osmiophilic body (UB) is also present in figure 32.

The studies carried out on different tumors in different species have revealed a number of similar findings. The behavior of various cell constituents has shown what migh be described as a reaction of the cells to the presence of the respective agents with many similarities and some differences. Perhaps further continued studies of this type, if combined with a number of other approaches, may in time yield a better understanding of the origin of other mammalian tumors in general and in particular of at least some human tumors.

Summary

The results are presented of electron microscope studies of thin sections of mammary tumors of mice, leukemic organs of mice and affected organs of chickens with visceral lymphomatosis.

Virus-like particles similar in appearance, but different in size have been found in the cells of the tissues examined. A number of characteristic changes in the cell constituents of the affected cells have been observed.

The observed changes in cells are similar in spontaneous leukemia and in leukemia induced in mice by cell-free extracts of leukemic organs. These changes also resemble those found in chicken lymphomatosis. Virus-like particles in chicken lymphomatosis are similar to those observed in Rous sarcoma.

Changes encountered in mouse leukemia and chicken lymphomatosis are also found in the cells of a cervical lymph node of a case of acute human leukemia. Virus-like particles are also present in the cells of the affected lymph node.

At present it is unknown what part, if any, is played by the virus-like particles in the origin of the tumors in the different species examined. The possibility has to be borne in mind that mice, chickens and humans may carry latent viruses which may for various reasons tend to localize in tumor tissues and play no part in the origin of the tumors.

Studies on the nature of the virus-like particles are now being carried out.

ACKNOWLEDGMENTS

Grateful acknowledgments for the excellent technical help rendered are due to Mr. Charles W. Lewis, Mrs. Ruth Durkee, Miss Mary Ashbrook, Miss Rose Marie Rienzi and Mr. Ben Rosario.

Discussion

Dr. Beard: Sometimes advances in science are hard to recognize and appreciate at the time, in many instances because of the conservatism and caution of investigators in reporting their results. Dr. Dmochowski came here this morning like a hot rodder, and he walked out like a lamb. I would have given my ears to have seen a thing of this sort done in our laboratory. Of course his caution is well-placed, but in my opinion this sort of thing has given us a foothold—a little rock to stand on to get us into a field that very much needs to be investigated. People have backed off from working with human beings because of the arduous difficulties in the field, and because of much antagonism toward the idea that cancer and malignancy in human beings might be virus induced. But now here is a means by which these things might be shown in human malignancy, and leukemia, if Dr. Goodpasture would call that a malignancy, and this is something to tie to. Now there is some place to work and to go on, and, personally, I am enormously appreciative of this finding.

Dr. Gross: I would like to ask if Dr. Dmochowski would comment a little bit as to whether he did see similar particles in normal leukemic mice, and then, if he would care, perhaps to give us some idea about possible differences in morphology between the mammary tumor agent and the mouse leukemia agent?

Dr. Dmochowski: I would like to answer the first question first. We did examine normal young mice of the same strains whose mice at a later age develop spontaneous leukemia. Negative findings in ultra-thin sections have to be considered with great reservations. If you do not find the thing that you are looking for in sections which constitute approximately one three-hundreth part of a cell, how can you record what is the statistical limit of the number of sections to be examined? How can you be sure that you have not encountered what you were looking for? On the

basis of such investigations that we have so far carried out, we have not encountered any of the changes or any virus-like particles in sections of normal organs of young mice of the same strain. This is in answer to the first question. Now for the second. There is a difference in morphology, although at a superficial glance it appears that there is little. First of all, in the case of the virus particles encountered in mammary tumors of mice, they are spherical and vary in size from around 500Å to 1,450Å; the virus particles encountered in chicken lymphomatosis and in Rous sarcoma are also spherical and vary in size from 700Å to 900Å. This statement requires reservation, unless you count several thousand particles, you can see for sure the extent of particle variation, and we only counted several hundred in the case of chicken lymphomatosis and Rous sarcoma virus. Therefore, this is only a preliminary statement. In the case of virus-like particles in mouse leukemia, both spontaneous and induced by cell-free extracts, they vary in size from 900Å to 1600Å; they appear more irregular in size and shape than those encountered in chicken lymphomatosis and Rous sarcoma. In the case of acute human leukemia, the virus-like particles are more spherical and are approximately 1000Å in diameter. This preliminary estimate of particle size may have to be corrected later, when more particles are measured.

Dr. DeCarvalho, Cleveland, Ohio: It was a surprise for me to see the structure in virus particles in your electron microscope pictures of human leukemic cells. I hope they were obtained in a state of good mood of your microscope because they may help me in understanding a cytopathogenic effect I obtained when I inoculated microsomes of human leukemic cells in human amnion cultures.

Dr. Melnick: I would like to say first that I am very much on the side of the speaker and the chairman in emphasizing how useful the electron microscope is in this field. However, I would like to take exception with the statement that the electron microscope provides a firm foundation on which to build. Particles are unlabelled in the electron microscope and I am sure because of the time limitation that Dr. Dmochowski referred to them as virus particles rather than as virus-*like* particles, because he had to say this over and over again, and it would have extended his talk.

We should remember that these particles which are seen within cells resemble virus particles, but the telling evidence to show a correlation between the particles and virus activity has not yet been made.

I would like to emphasize another point. Even in cells that are definitely known to be infected with viruses, as with polio or measles virus infected cells, where we can measure with a fairly high degree of accuracy the number of infectious particles present, it is very difficult to find anything that we can correlate with the virus-like particles, such as those which Dr. Dmochowski showed.

It seems to me crucial in this work to have some correlative measurement at the time that the particles appear in cells. There must be some kind of biological or other assays system which can be used, in order to have this foundation really be as firm as we all would like it to be.

Dr. Goodpasture: Dr. Melnick has made my speech. Back in the early days of investigating the etiology of infectious disease there were certain postulates, that served as precautionary measures in trying to determine whether or not a particular specific morphological or cultural agent was really the cause of the disease under investigation. Now, if we are to be told that some little particle that we see in the cytoplasm of a cell is a virus, without going into any other exhibition of evidence that such a thing is infectious, I think we had better exercise a little more caution; more caution even than calling a tumor or cancer a hyperplasia that is induced by an infectious agent.

Dr. Beard: I would like to see this go on for a long time, but I think we had perhaps better go ahead. I just want to remind Dr. Goodpasture that you see, that we're just small boys trying to get along. He knows so much, and we know so little, but we're just going to keep on struggling anyhow. I still say that this is a rock to stand on, and we're going to stand on it.

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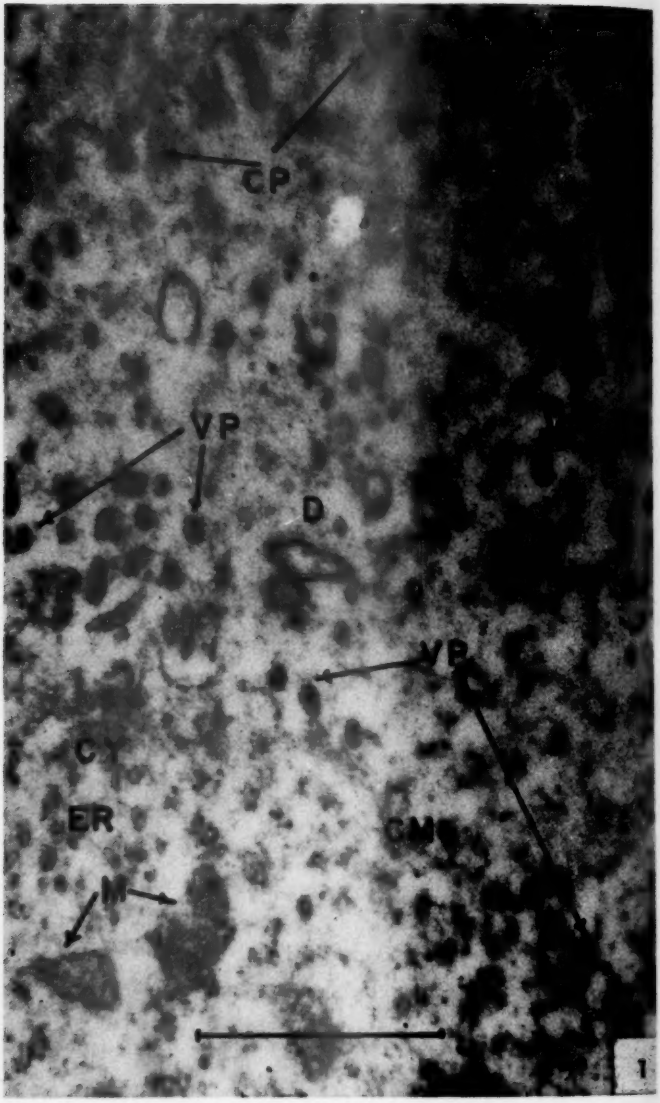

FIG. 1. Cells of a lung metastasis of a high-cancer-strain mammary tumor. Cytoplasmic processes (CP); cellular membranes (CM); mitochondria (M); cytoplasm (CY). Virus-like particles (VP) in a duct (D). x36,000.

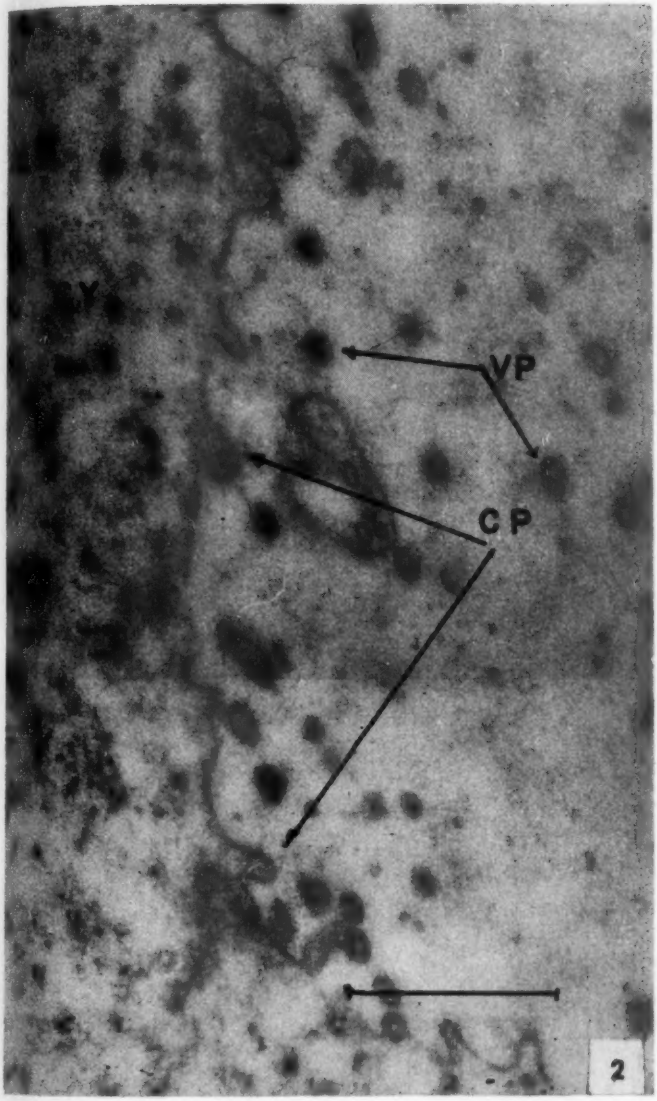

FIG. 2. Edge of a cell of a metastatic tumor in the lung of a virus-carrying mouse. Part of a duct is also seen. Cytoplasm (CY); cytoplasmic processes (CP); virus-like particles (VP). x40,000.

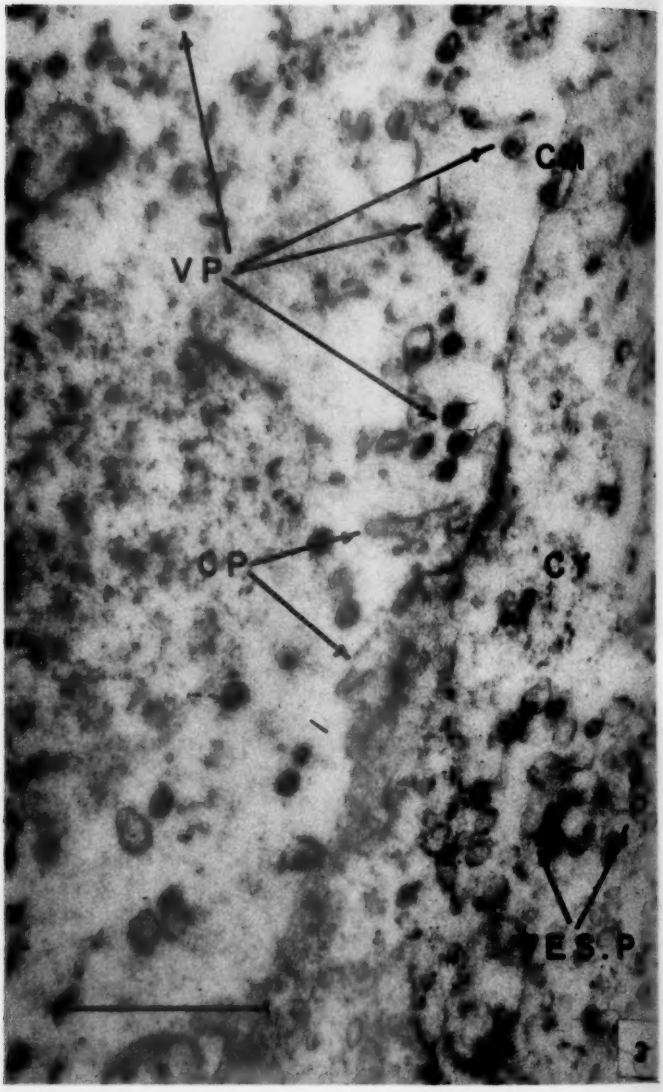

FIG. 3. Cells of an apparently virus-free mammary tumor. Cytoplasm (CY); vesicular type particles (VESP); cytoplasmic processes (CP); cellular membrane (CM); virus-like particles (VP). x40,000.

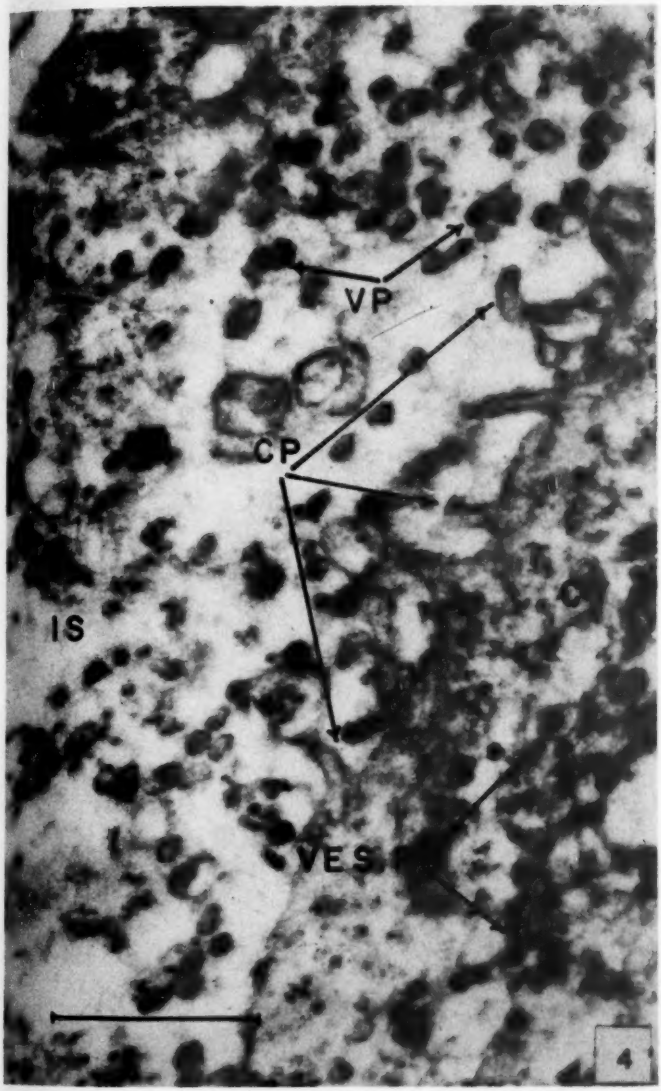

FIG. 4. Low-cancer-strain apparently virus-free strain mammary tumor. Cytoplasm (CY); vesicular type particles (VESP); cytoplasmic processes (CP); virus-like particles (VP) in an intercellular space (IS). x40,000.

FIG. 5. Normal cells of a cervical lymph node in spontaneous mouse leukemia. Nuclei (N); nucleoli (NU); cytoplasm (CY); mitochondria (M). x10,000.

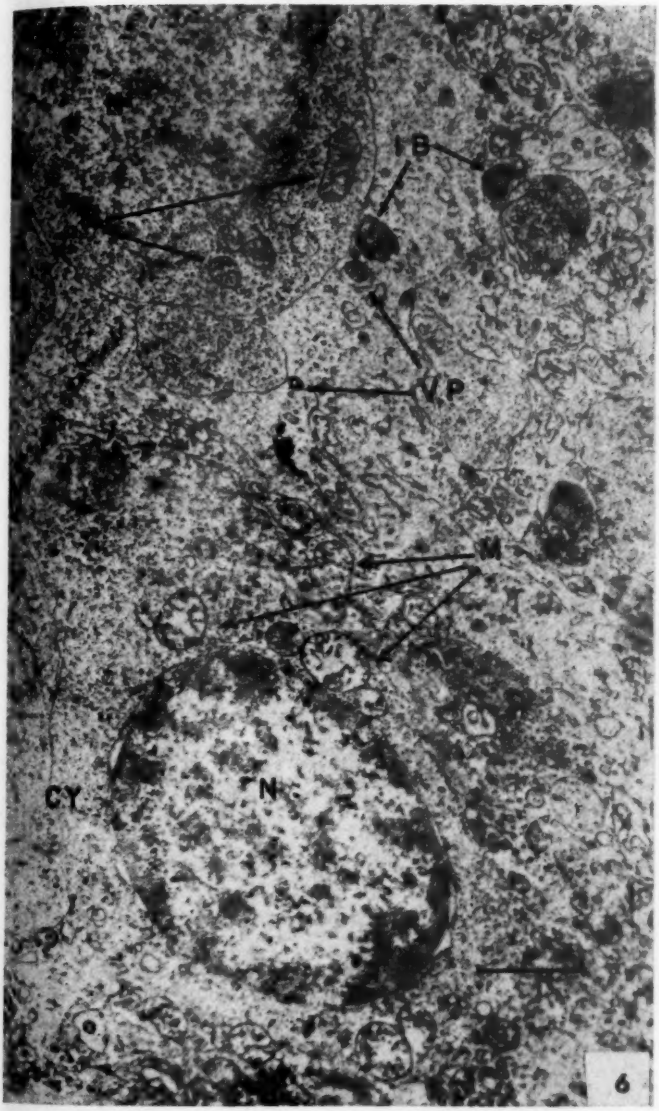

FIG. 6. Affected cells of cervical lymph node in spontaneous leukemia of mice. Nuclei (N); cytoplasm (CY); mitochandria (M); inclusion bodies (IB); virus-like particles (VP). x15,000.

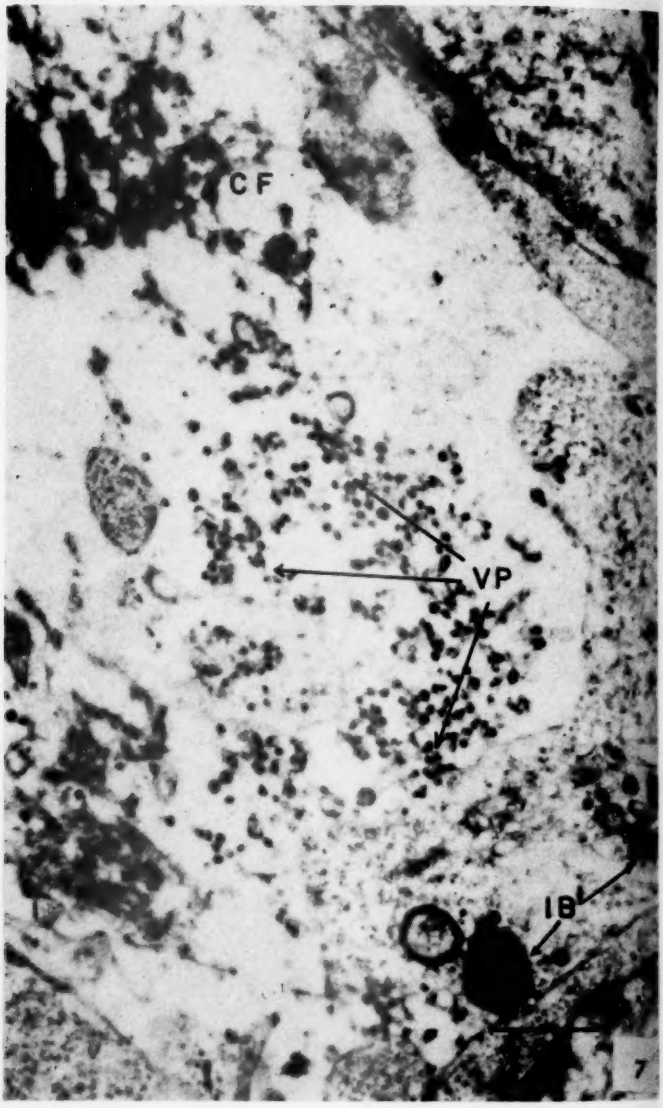

FIG. 7. Spontaneous mouse leukemia. Cervical lymph node. Cellular fragments (CF); virus-like particles (VP); inclusion bodies (IB). x18,000.

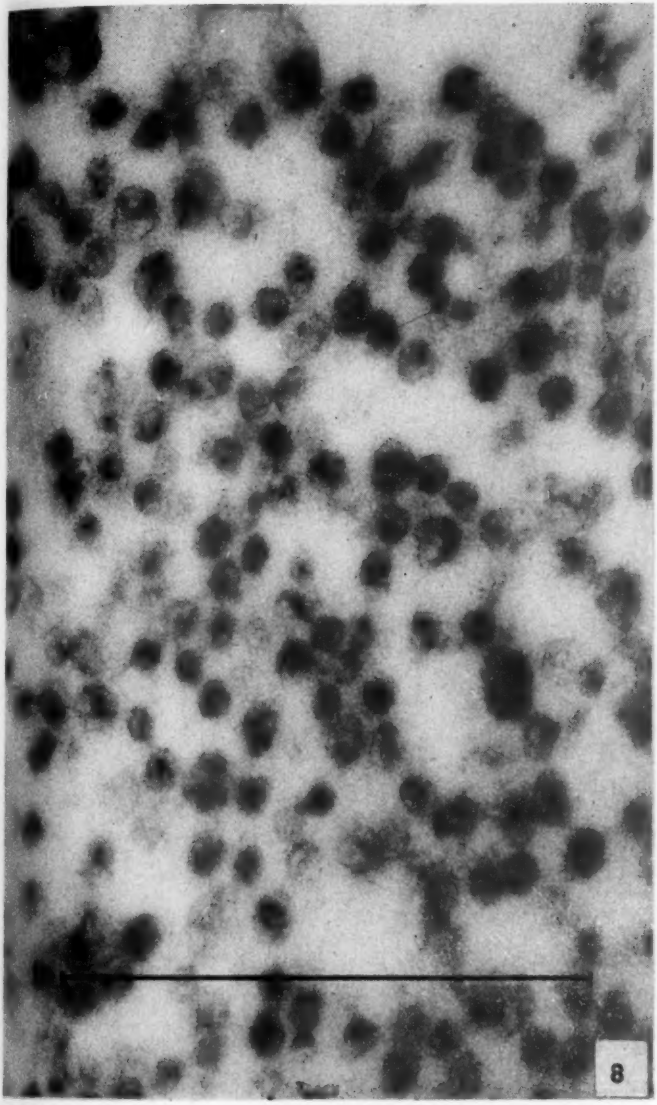

FIG. 8 Spontaneous leukemia in mice. Internal structure of virus-like particles. x77,000.

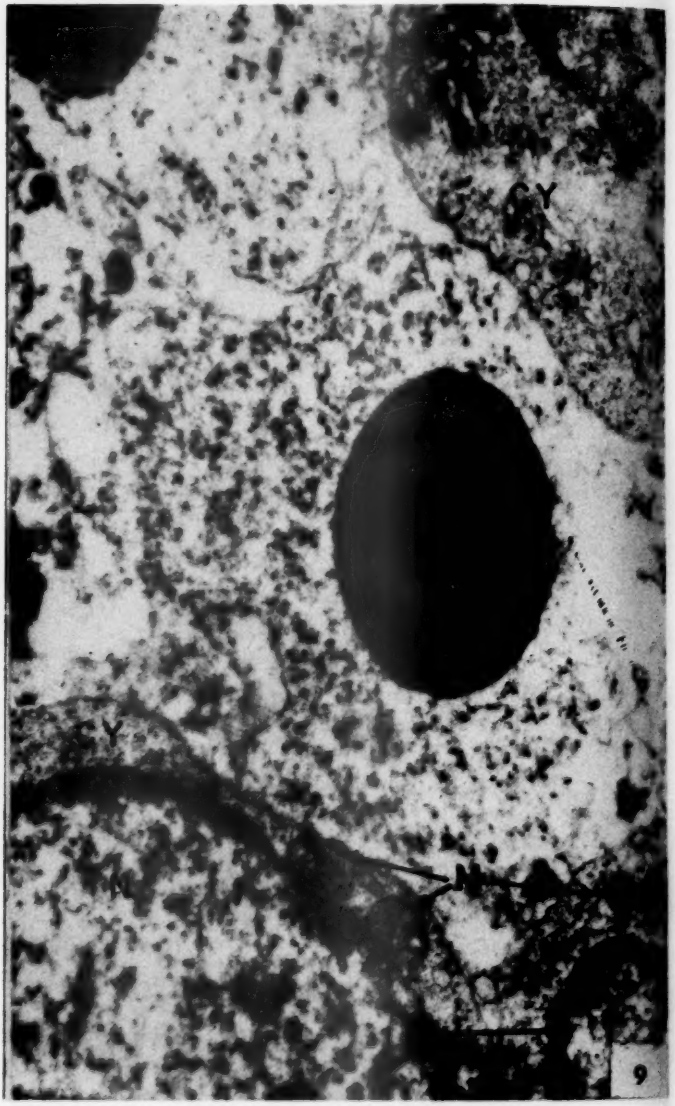

FIG. 9. Induced leukemia of mice, thymus gland. Nuclei (N); cytoplasm (CY); mitochondria (M); unknown osmiophilic body (UB) among cell fragments. x18,000.

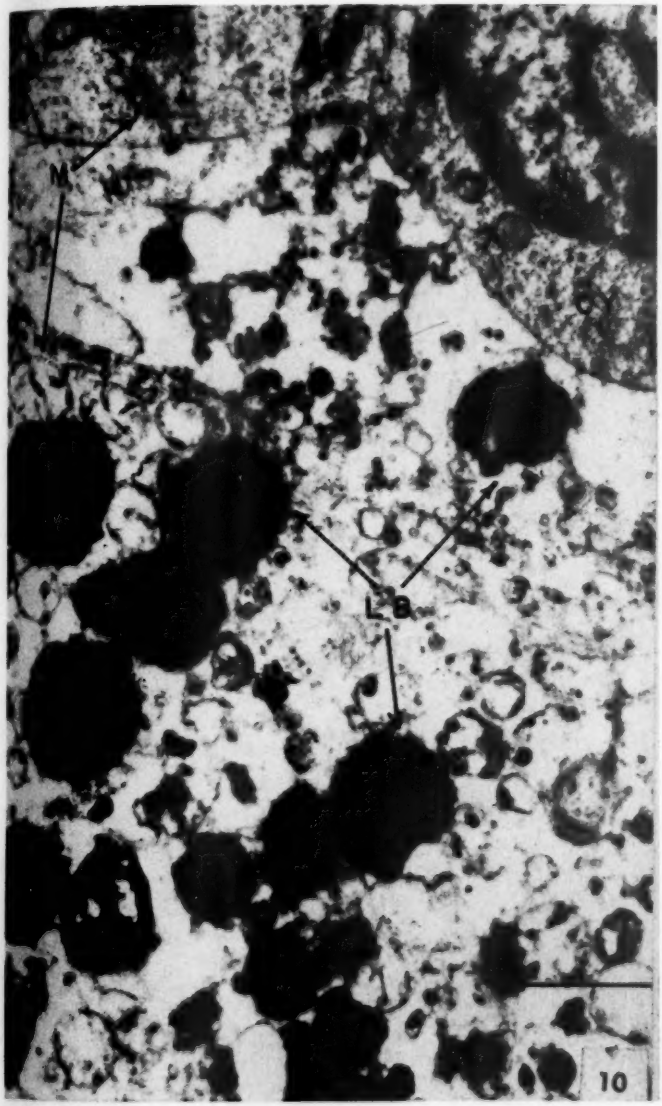

FIG. 10. Induced leukemia of mice. Thymus gland. Nuclei (N); cytoplasm (CY); mitochondria (M); lipid body (LB) in degenerating cytoplasm. x18,000.

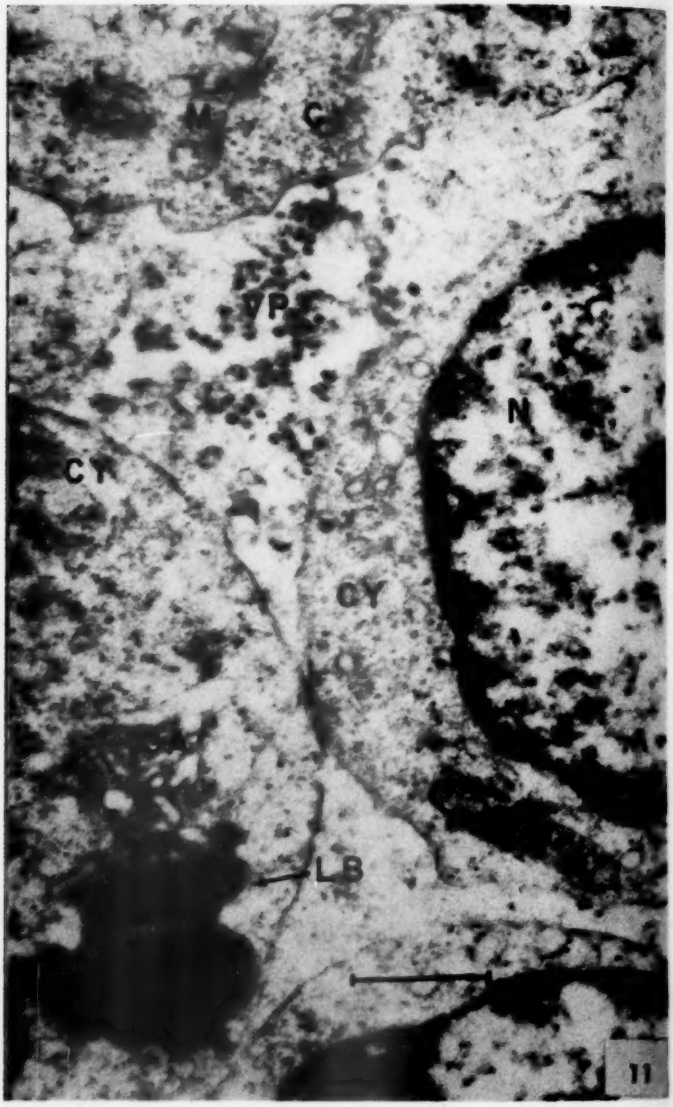

FIG. 11. Thymus gland in induced leukemia of mice. Cells with nuclei (N); cytoplasm (CY) with mitochondria (M), Golgi apparatus (GA) and lipid body (LB). Virus-like particles (VP) in the intercellular space. x22,000.

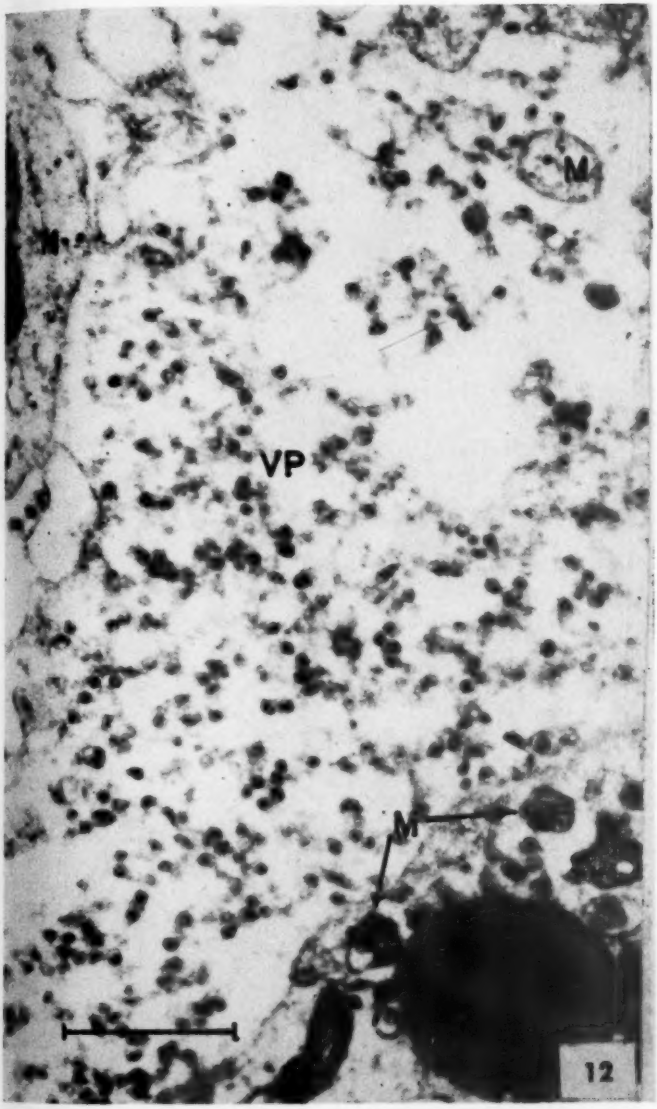

FIG. 12. Induced mouse leukemia. Thymus gland. Nuclei (N); cytoplasm (CY) with mitochondria (M). Virus-like particles (VP) in large numbers. Unknown body (UB). x25,000.

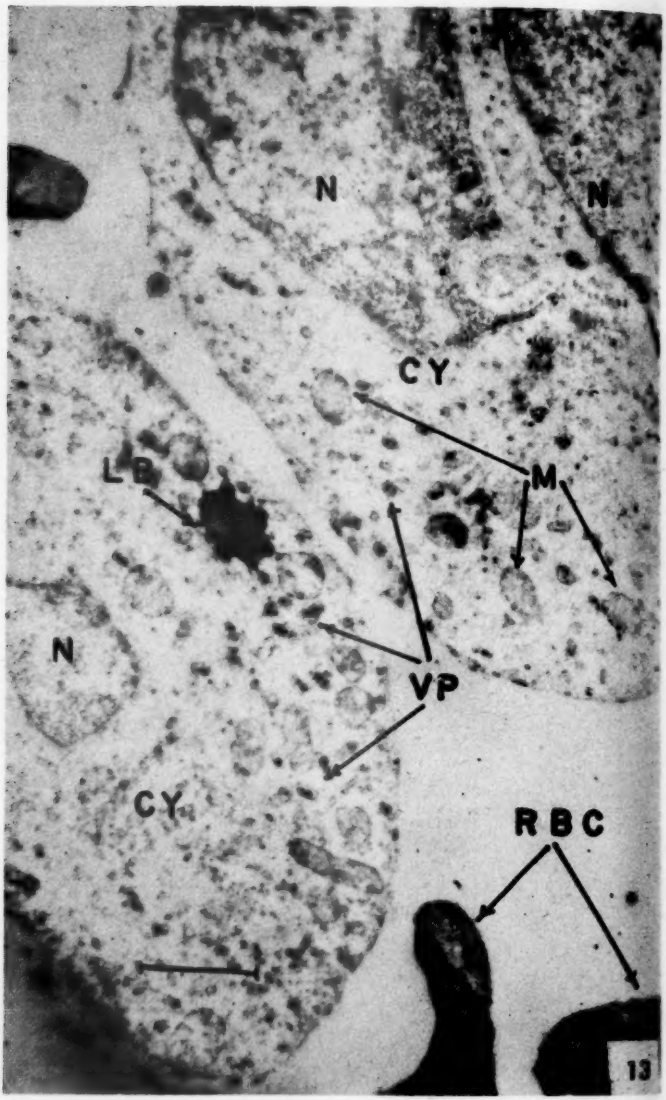

FIG. 13. Granulocytic leukemia in mice. Ascites form of transplanted leukemia. Nuclei (N); cytoplasm (CY) with mitochondria (M) and single virus-like particles (VP) and lipid body (LB). Red blood corpuscles (RBC). x18,000.

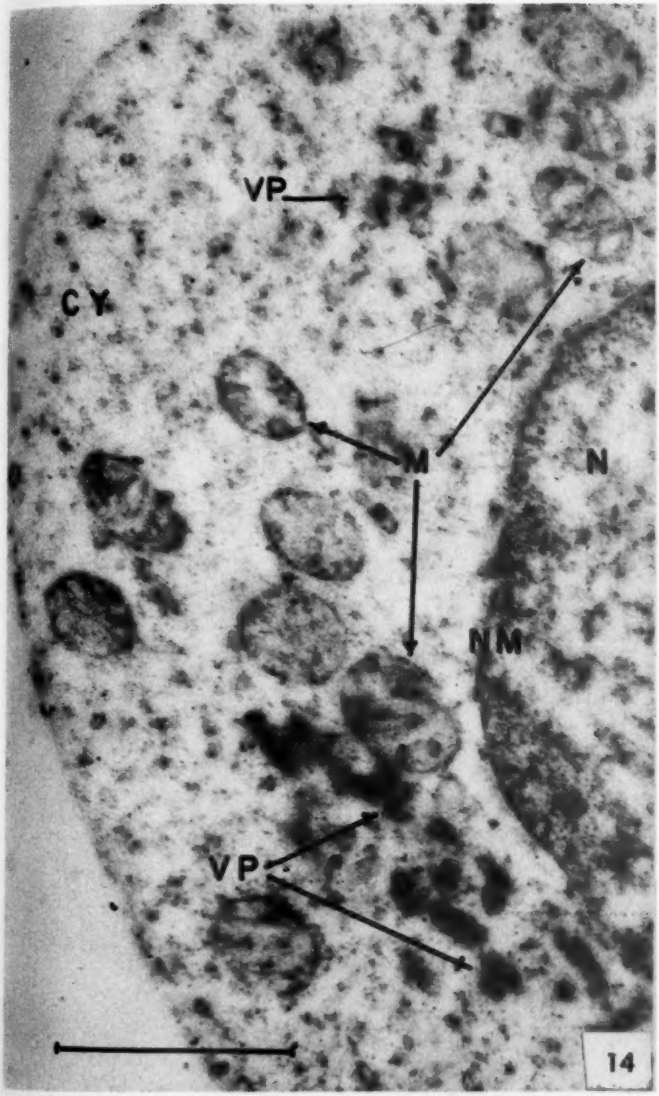

FIG. 14. Granulocytic leukemia in mice. Ascites form of transplanted leukemia. Nucleus (N) with nuclear membrane (NM). Cytoplasm (CY) with mitochondria (M) and virus-like particles (VP) within endoplasmic reticulum or ergastoplasm. x35,000.

FIG. 15. Intravascular visceral lymphomatosis of chickens. Comparatively unchanged liver. Cytoplasm of cells (CY) shows mitochondria (M) and cytoplasmic processes (CP) extending in a biliary duct, along the cell membranes (CM). x25,000.

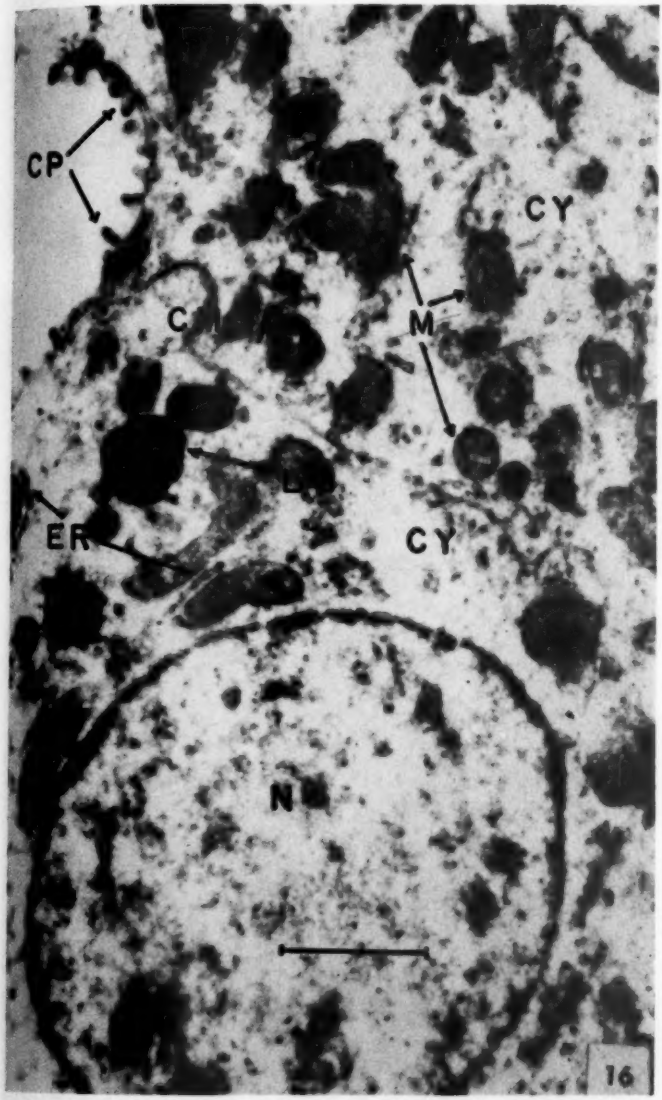

FIG. 16. Liver in intravascular visceral lymphomatosis of chickens. Cells showing nuclei (N) cytoplasm (CY) with mitochondria (M) and ergastoplasm (ER) characteristically surrounding the mitochondria. Cellular membranes (CM) with cytoplasmic processes (CP). Lipid bodies (LB) are present. x21,000.

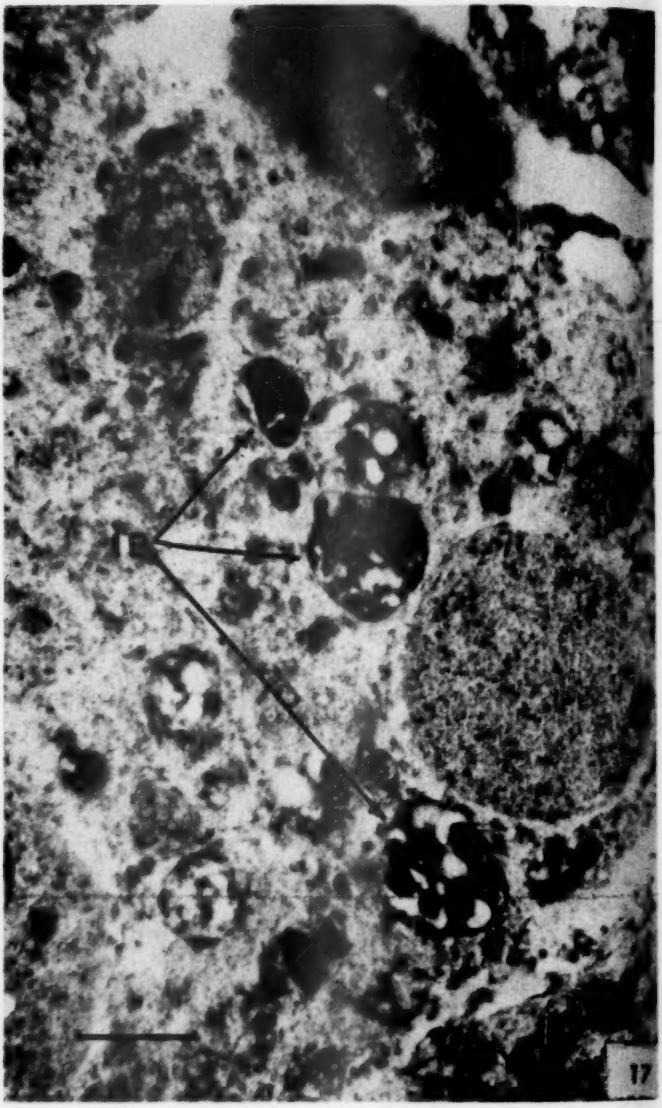

FIG. 17. Liver in intravascular visceral lymphomatosis of chickens. Cytoplasm (CY) showing inclusion bodies (IB). x18,000.

FIG. 18. Extravascular visceral lymphomatosis of chickens. Liver showing comparatively normal cell and a cell undergoing degeneration. Nucleus (N) with nucleoli (NU), cytoplasm (CY). Cytoplasm of another cell with vacuoles (V) and inclusion bodies (IB) showing virus-like particles (VP). x18,000.

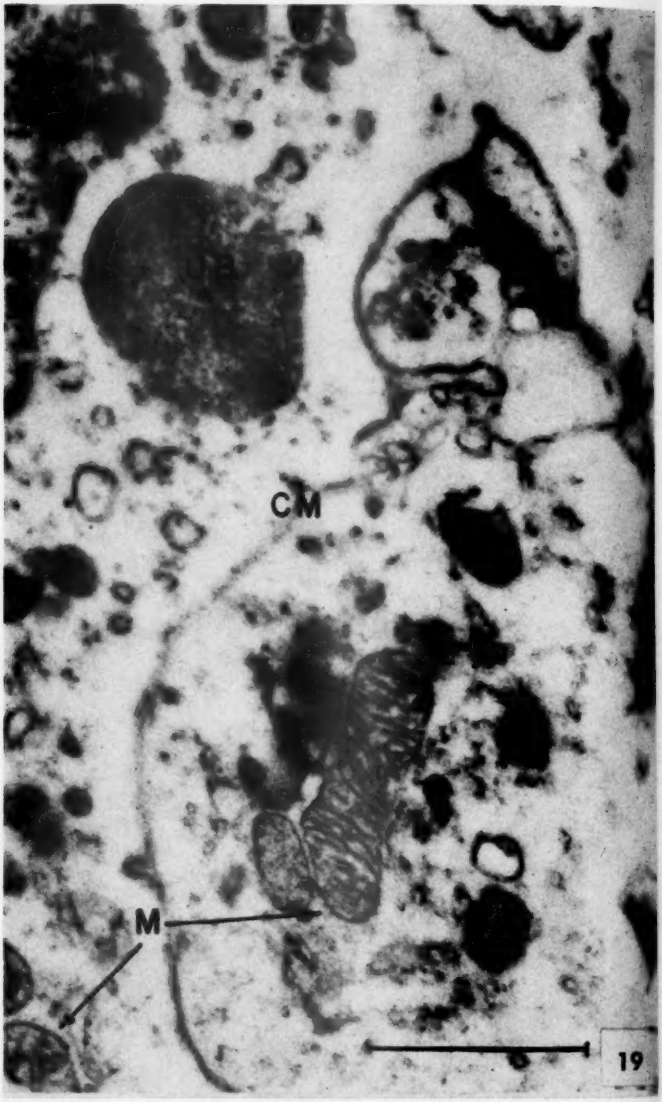

FIG. 19. Visceral lymphomatosis of chickens. Cells of spleen showing mitochondria (M), cellular membranes (CM), unknown body (UB) and nucleus (N). x32,000.

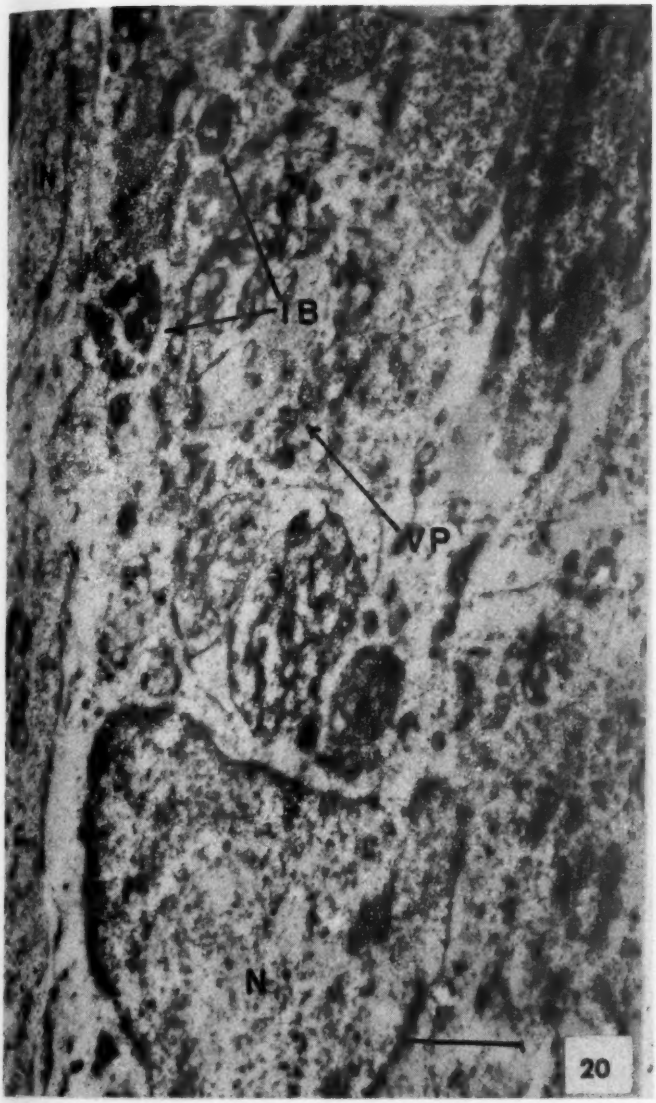

FIG. 20. Spleen in extravascular visceral lymphomatosis of chickens. Cells showing nuclei (N) and their cytoplasm inclusion bodies (IB) and virus-like particles (VP). x18,000.

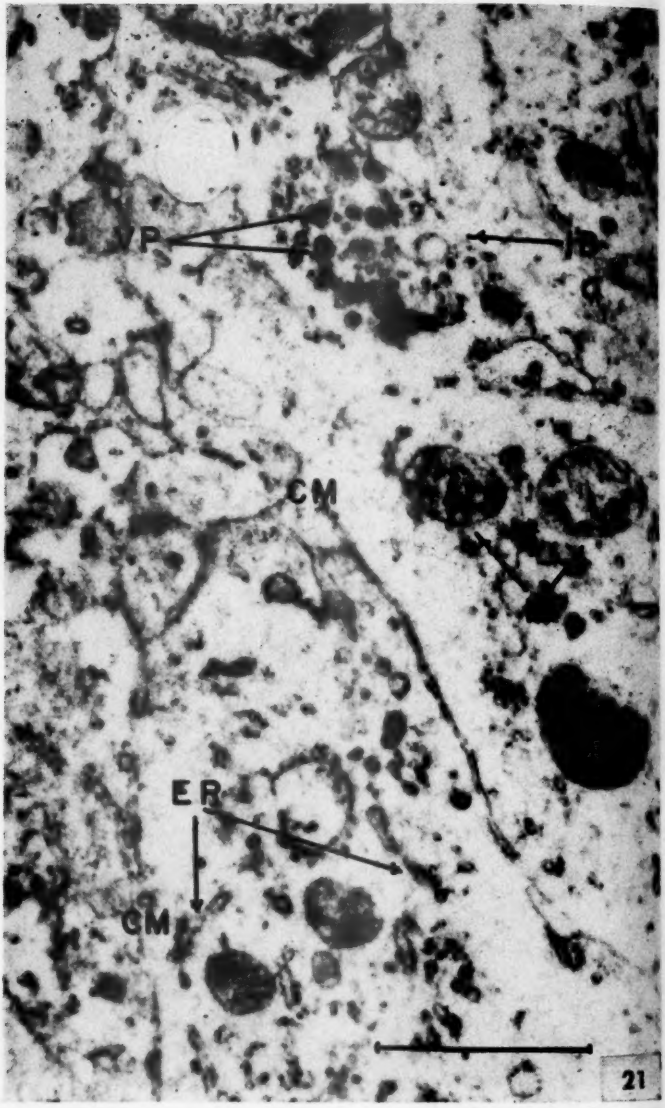

FIG. 21. Spleen in intravascular visceral lymphomatosis of chickens. Cells separated by cellular membranes (CM) showing mitochondria (M), ergastoplasm (ER), an unknown body (UB) and inclusion body (IB) with virus-like particles (VP). x32,000.

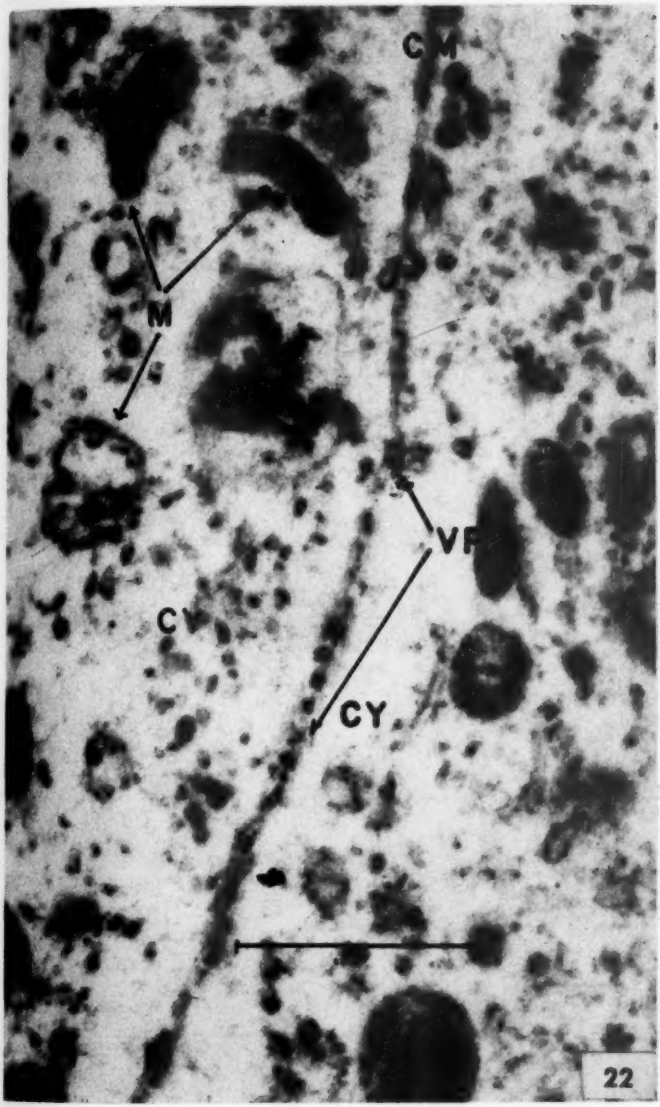

FIG. 22. Spleen in intravascular visceral lymphomatosis of chickens. The cytoplasm (CY) of two cells separated by cellular membranes (CM) with virus-like particles (VP) lying between the membranes. Mitochondria (M) in various stages of degeneration. x36,000.

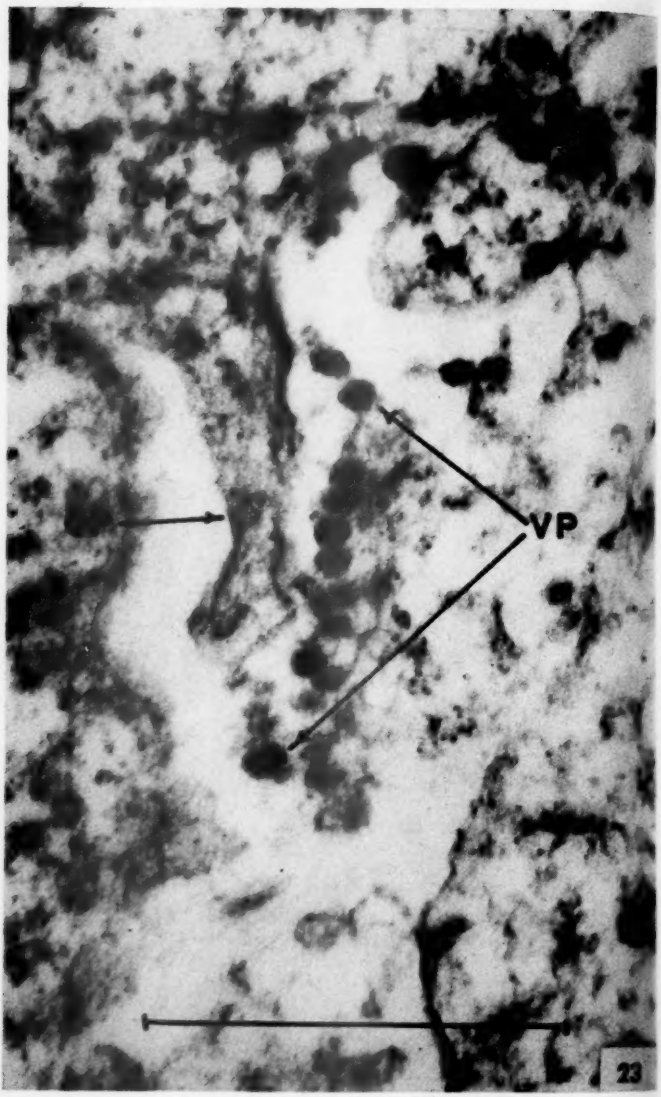

FIG. 23. Intravascular visceral lymphomatosis of chickens. Spleen. Virus-like particles (VP) lying along a cytoprasmic process (CP) of the cytoplasm (CY) of one of the cells. x63,000.

FIG. 24. "Hot" passage of Rous sarcoma virus of chickens. Tumor of left wing. Cell showing mitochondrion (M) and ergastoplasm (ER). x32,000.

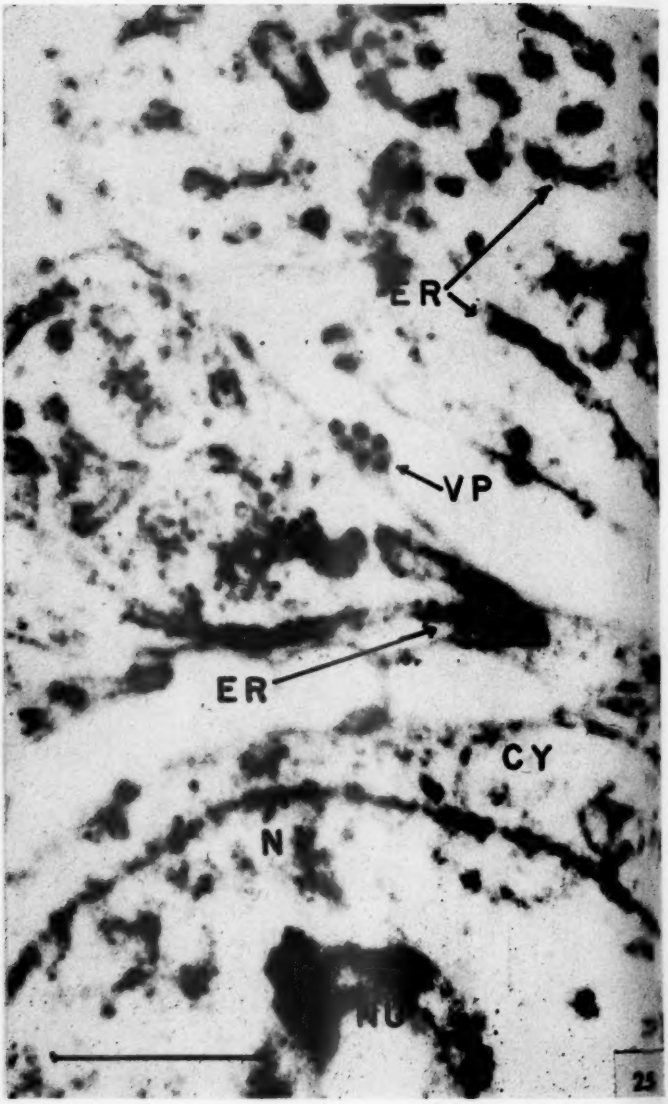

FIG. 25. "Hot" passage of Rous sarcoma virus of chickens. Tumor of left wing. Cell showing nucleus (N) with nucleolus (NU). Cytoplasm (CY) of some cells showing ergastoplasm (ER) and a group of virus-like particles (VP) on the outside of a cell membrane (CM). x32,000.

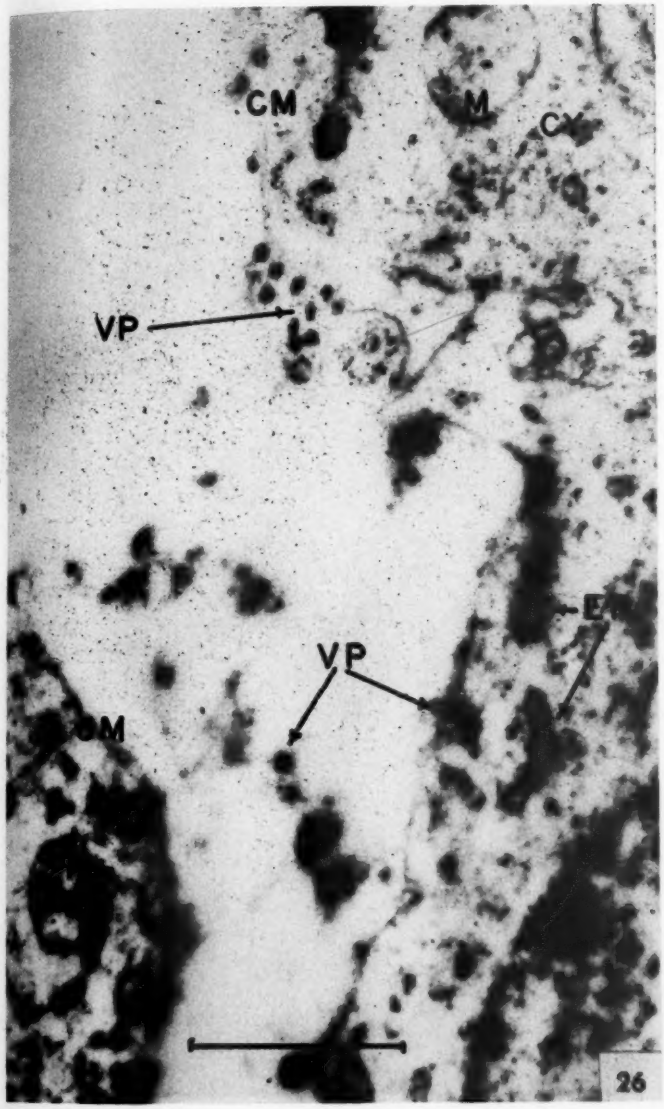

FIG. 26. "Hot" passage of Rous sarcoma virus of chickens. The cytoplasm of cells (CY) with ergastoplasm (ER) and mitochondria (M) undergoing change. Virus-like particles (VP) in groups, pairs or single present outside the cell membrane (CM). x32,000.

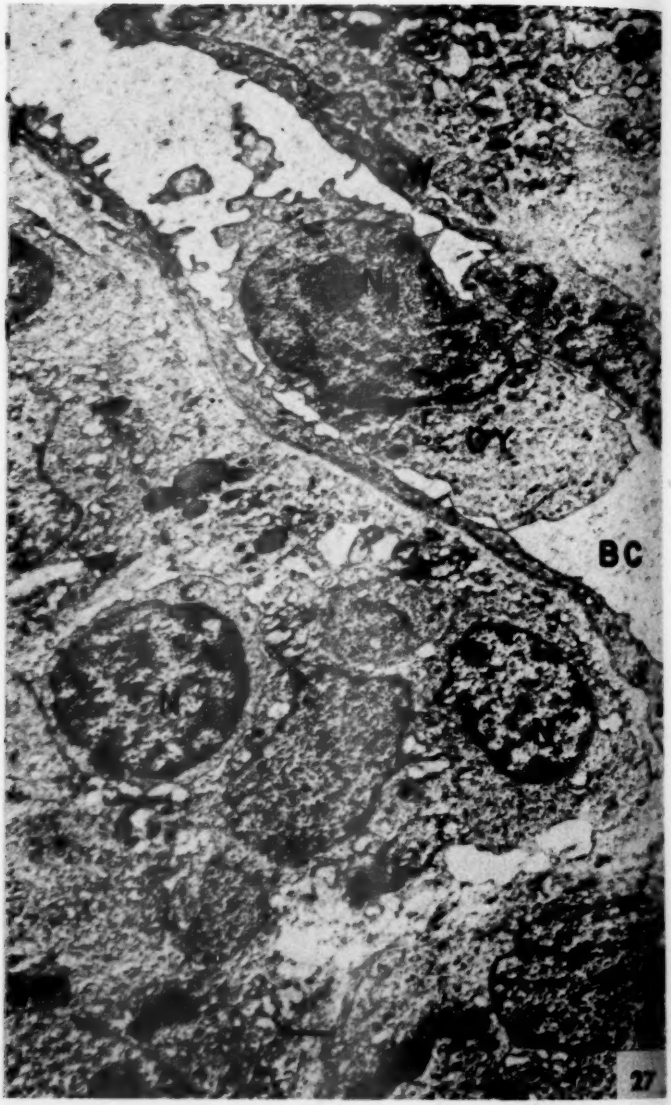

FIG. 27. Human acute lymphatic leukemia. Cervical lymph node showing a blood capillary (BC) with capillary wall (CW) and a white blood cell in which the nucleus (N) and cytoplasm (CY) can be seen. Cells showing nuclei (N) comparatively unchanged. x7,000.

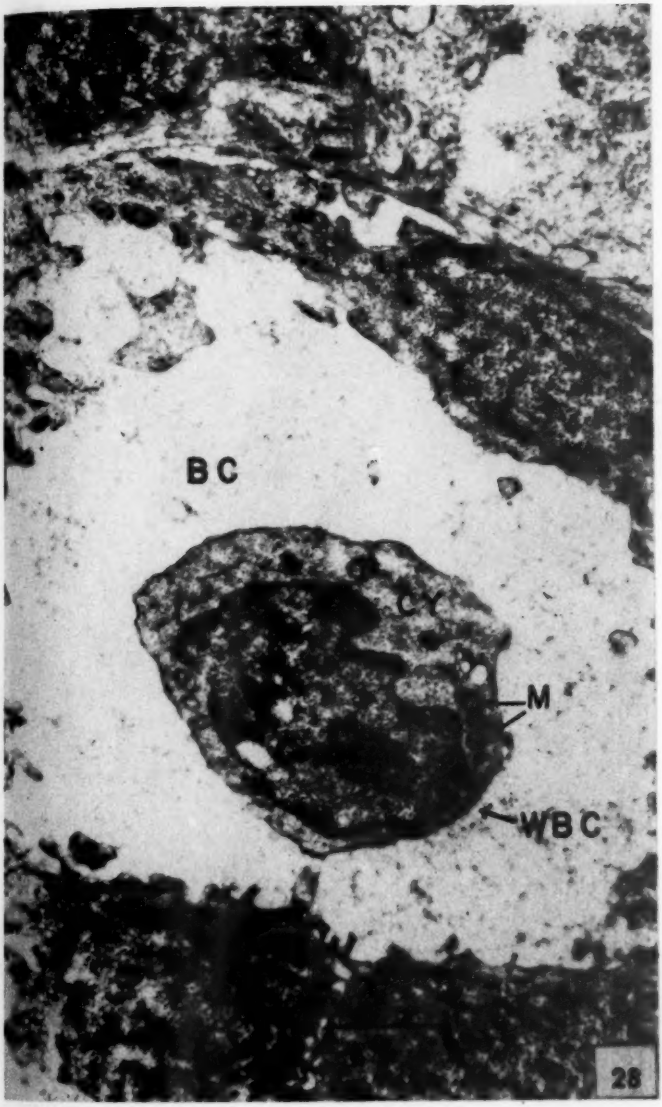

FIG. 28. Human acute lymphatic leukemia. Blood capillary (BC) showing in a cross-section the capillary wall and a white blood cell (WBC) in its lumen. Nucleus (N), cytoplasm (CY) and mitochondria (M) can be seen in the cell. x10,000.

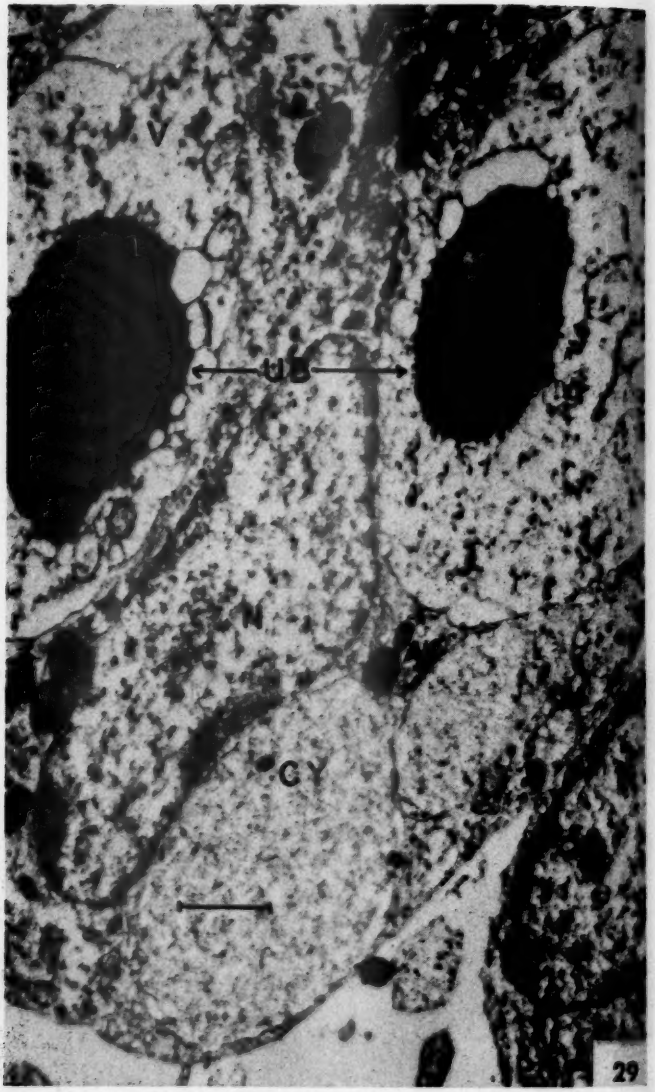

FIG. 29. Human acute lymphatic leukemia. Cytoplasm (CY) of cells of a lymph node showing vacuoles (V), formation of unknown osmiophilic bodies (UB) and nuclei (N). x14,000.

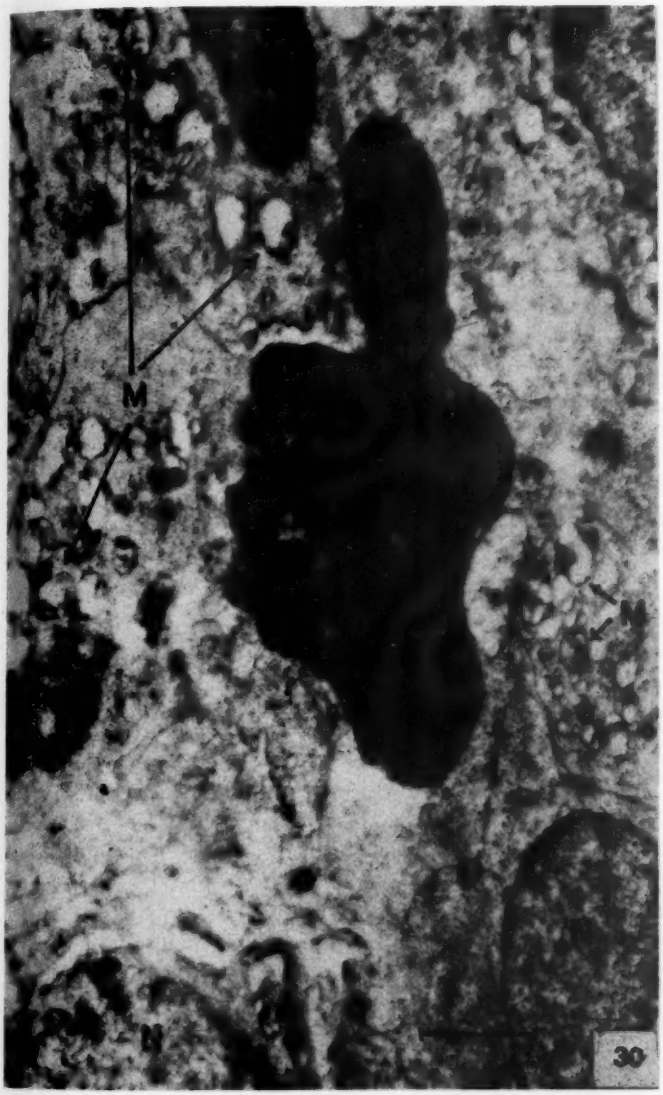

FIG. 30. Cells of a cervical lymph node in human acute lymphatic leukemia undergoing changes. Nuclei (N), the cytoplasm with mitochondria (M) undergoing breakdown and a large unknown body (UB). x18,000.

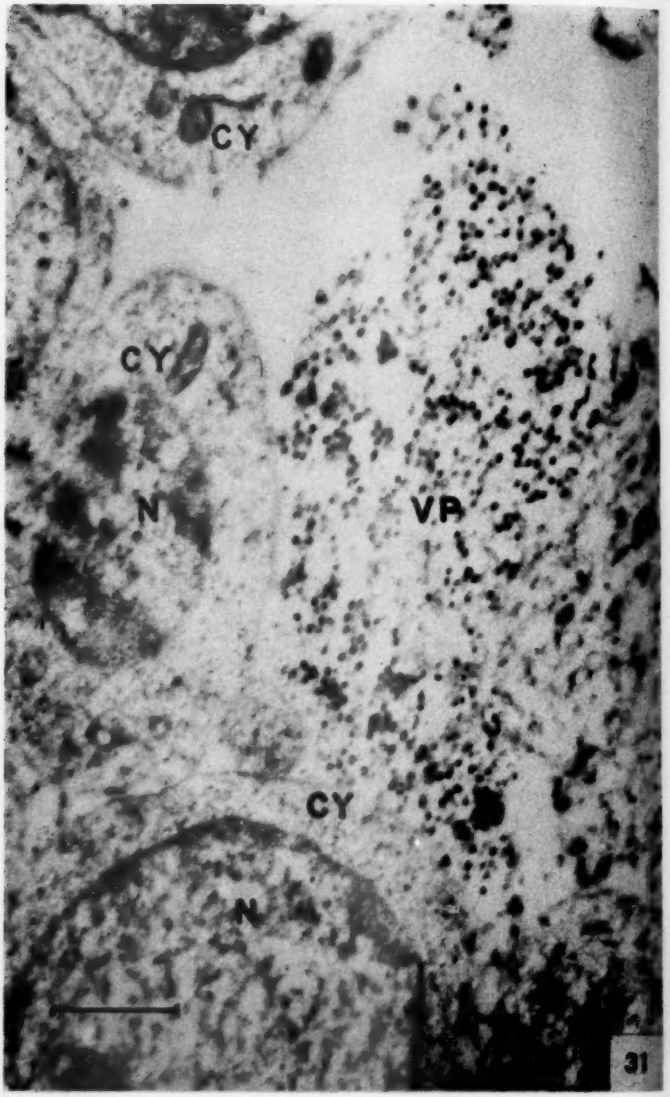

FIG. 31. Virus-like particles (VP) in an intercellular space of a cervical lymph node in the case of human acute leukemia. Cells comparatively unchanged showing nuclei (N) and cytoplasm (CY) with mitochondria (M). x18,000.

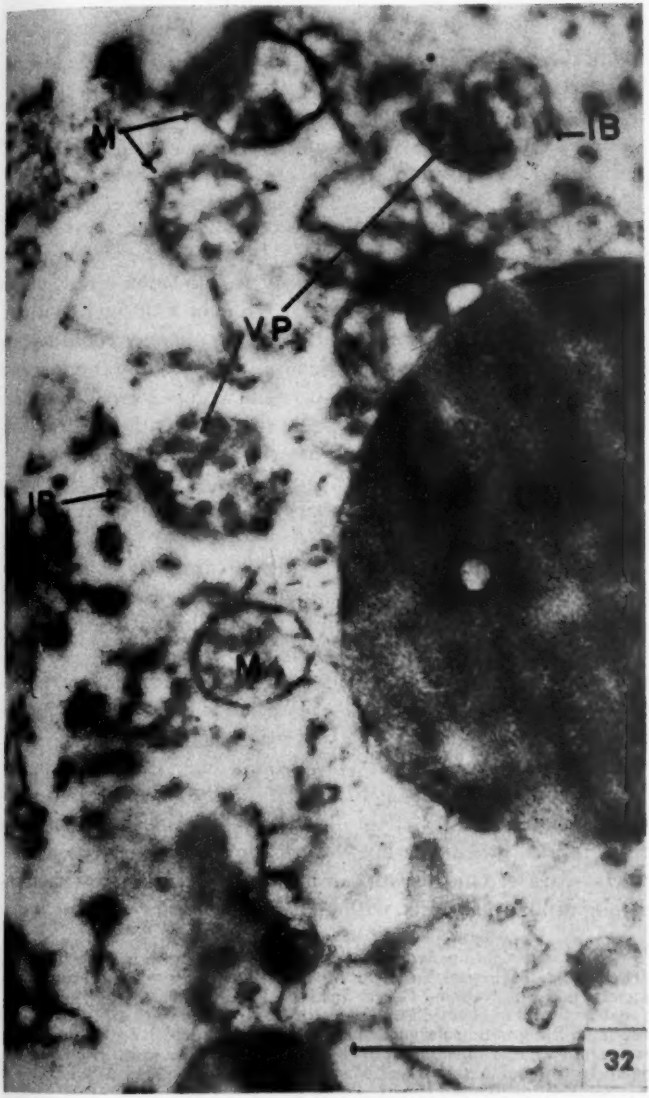

FIG. 32. Part of cytoplasm of a cell of the cervical lymph node in human acute leukemia. Large osmiophilic unknown body (UB); mitochondria (M) undergoing degeneration. Inclusion-like bodies with virus-like particles (VP) present. x35,000.

STUDIES ON THE COMBINED EFFECTS OF CHEMICAL CARCINOGENS, HORMONES AND VIRUS INFECTION*

F. DURAN-REYNALS†

It has been established that cancers induced by treatment with carcinogens, in a given host, largely look and behave like similar types of cancers occurring naturally in the same host. Since a number of the latter cancers are known to be induced by viruses, it seemed logical to assume that the same etiology could be postulated for those cancers experimentally induced by carcinogens. And if such should be the case, it would also be logical to assume that these cancer viruses, brought into activity by the carcinogen, are latent in the host and are revived by a nebulous "trigger" effect, although no more nebulous than the effects that bring out a latent herpes infection or even a dormant tubercular process.

A further, but not indispensable, assumption concerns the nature of the latent viruses brought out by the carcinogen. Would they be viruses that *always* behave as cell stimulating, neoplastic viruses? Or, could they be — at least in some cases — viruses that under ordinary circumstances induce inflammatory, necrotizing lesions, but that under the circumstances created by the effect of carcinogens, induce neoplastic lesions?

This supposition first appeared plausible to us when it was found (Duran-Reynals, 1940) that typical cancer viruses, those of the avian sarcomas of the Rous type, injected into immature hosts induce no cancer but destructive conditions: the hemorrhagic disease. This disease can be kept indefinitely, by passages through immature hosts, without a single cancer cell being found

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† Department of Microbiology, Yale University School of Medicine, New Haven, Connecticut.

in the lesions. Yet, when extracts of these lesions are injected into older hosts, typical sarcomas develop.

This observation, which has been confirmed by several investigators, naturally led to the reflection that if typical cancer viruses can behave as ordinary viruses, the opposite could perhaps be true. This working hypothesis was further supported, in our mind, by the following considerations already summarized in a recent publication (Duran-Reynals, 1957b).

A. In what concerns their composition, morphology, antigenicity, epidemiology, etc., one finds the same properties, and

FIG. 1. Hemorrhagic disease in a duckling injected intravenously at the age of 1 day with the virus of duck variant 14(d)7 of the Rous chicken sarcoma.

FIG. 2. Sarcoma resulting from subcutaneous injection, in a duck 3 weeks old, of an extract of the hemorrhagic lesions induced by injection of virus of duck variant 14(d)7 in a younger host.

in the same degree of variability, in neoplastic as in ordinary viruses.

The one basic difference between these two groups of viruses is that the clinical lesions, *as usually perceived by the observer*, consist either of cell stimulation or of cell destruction. This difference, however, may exemplify the extremes in a gradation of compatibility between virus and cell; indefinite cell stimulation thus would be the result of perfect compatibility between virus and cell, and cell destruction would result from total incompatibility between virus and cell. Between these two extremes one

would have to postulate intermediate states of compatibility between virus and cell, which is precisely the case, as follows:

B. Viruses endowed with the property of inducing cell destruction can also induce cell stimulation ranging from:

Temporary cell stimulation, *e.g.*, pox viruses;

Longer lasting cell stimulation, *e.g.*, rabbit fibroma;

Indefinite, histologically benign, cell stimulation, *e.g.*, sheep pulmonary adenomatosis;

Indefinite, malignant cell stimulation, *e.g.*, avian sarcomas.

The cell stimulating effect is conditioned, in the whole or in degree, by:

Variation of the virus, *e.g.*, several pox diseases, rabbit fibroma;

Variation of the virus plus age of the host, *e.g.*, sheep pox, rabbit fibroma;

Age of the host, *e.g.*, avian sarcomas;

Phase of the disease, stimulation of the cell preceding its necrosis, *e.g.*, pox viruses or, following it, as is probably the case in sheep pulmonary adenomatosis, sheep pox and still others.

The last consideration could perhaps be further elaborated as follows: In general, infection by ordinary viruses, and by other agents as well, occurs so much more often in the young host that one may wonder why — as best shown in the case of birds and mice — infectious malignancy should occur in adult age, that is cancer age. This apparently anomalous situation could theoretically be explained on the basis that the infection does indeed occur in the immature host, but that it does not become clinically manifest, in its neoplastic phase, until the animal reaches maturity.

Chicken Sarcomas

We shall first discuss these tumors because it was in chicken sarcomas, chemically induced, that viruses, as possibly mediating the effects of the carcinogens, were first reported. Causative viruses in spontaneous chicken sarcomas were discovered in 1912 by Rous and Murphy; why, then, should they not be found in tumors morphologically and functionally so similar, as those resulting from the injection of carcinogens into the muscles of chickens? The reasoning seems more forceful if the chemically induced tumors are compared, not only to those of the Rous type, but also to fibrosarcomas such as chicken tumor C (Duran-Rey-

FIG. 3. Tumor induced in a chick by inoculation of cells of a methylcholanthrene induced sarcoma. This tumor proved to be indefinitely transplantable, but attempts to show a causative virus failed.
FIG. 4. The same tumor as in Fig. 3 transplanted into a duckling.

nals and Shrigley, 1946). For, like most of the chemically induced sarcomas, chicken tumor C is rather firm, lacks viscidity, grows rather slowly, and metastasizes but rarely; causative virus in chicken tumor C can only be detected in tumors growing in young hosts, and even then the filtrates are active for only a third of the chicks injected; the virus never induces hemorrhagic lesions.

The presence of a causative virus in the chemically induced sarcomas was affirmed, in the early twenties, by Carrel, to be later denied by Murphy and others. Still later, in England, the presence of such viruses was again reported by McIntosh and Selbie, but denied by Peacock, while, in France, they were reported once more by Oberling and Guerin. On the other hand, indirect immunological evidence favoring the existence of causa-

tive viruses in these tumors was reported by Andrewes, Dmochowski, and Foulds.

On and off for several years we also tried to detect causative viruses in the chemically induced tumors, keeping in mind that such viruses could either manifest themselves by inflammation and necrosis, or by neoplasia. Since spontaneous chicken tumors, both of the Rous sarcoma or of the chicken tumor C type, often become non-filterable when growing in mature hosts, we used young hosts both for the induction and for the transplantation of tumors, to avoid this masking effect of the age factor on the hypothetical virus. This was done by injecting 1 mg. of m.c. into chicks generally one month old — younger birds frequently perishing from acute effects of the chemical — and by transplanting cell suspensions of the tumors, as the growths attained a sizeable dimension, into other chicks in successive passages. Sarcomas were induced also in older hosts by injecting larger amounts of m.c., and the transplantability of these tumors was similarly investigated. A total of 84 tumors were studied. Results

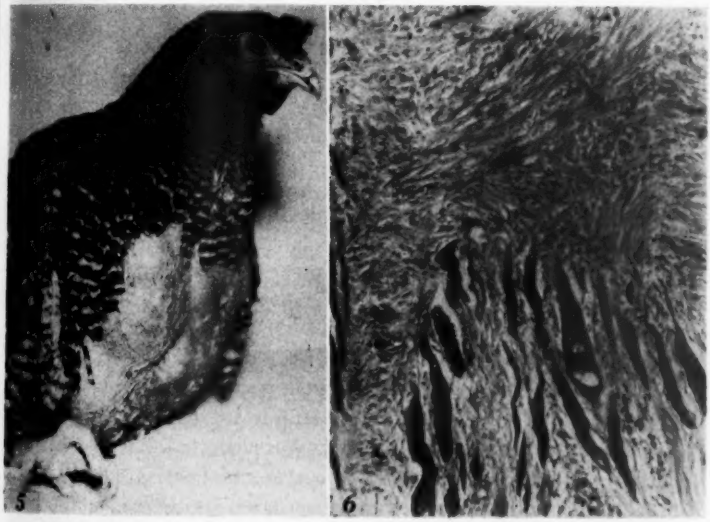


FIG. 5. Chicken tumor C after 6 months of growth in a chicken inoculated at the age of 6 weeks.
FIG. 6. Microscopic aspect of chicken tumor C. Magnification X 230.

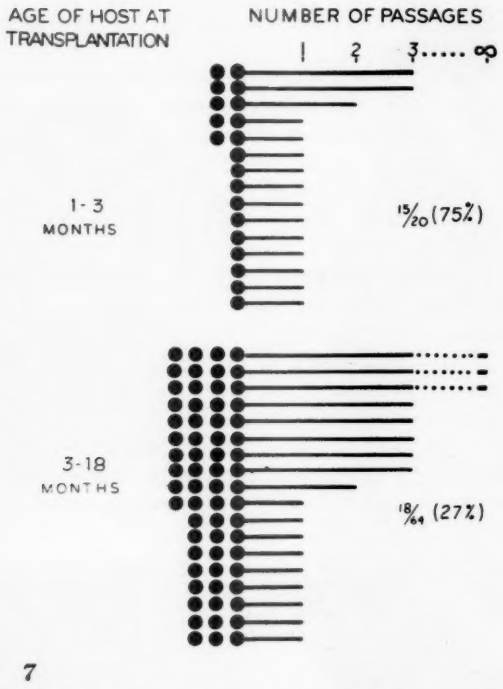


FIG. 7. Transplantability into chicks of methylcholanthrene induced tumors from chickens of different ages. Each circle represents a tumor tested, and the lines emanating from the circles measure the number of successful cell passages.

show that 15 out of 20 sarcomas (75 per cent) secured from hosts aged from 1 to 3 months were transplantable, whereas such event occurred only in 18 out of 64 sarcomas (28 per cent) secured from hosts aged from 3 to 18 months. However, transplantation of the tumors from the younger age group never went beyond a third passage, the lines being lost in every case despite the fact that large numbers of chicks were injected. Paradoxically perhaps, the only 3 tumors that became indefinitely transplantable came from hosts of the older age group. One may add that transplantability reached its minimum beginning in the tumors from hosts aged 3 months, to remain stationary until the hosts

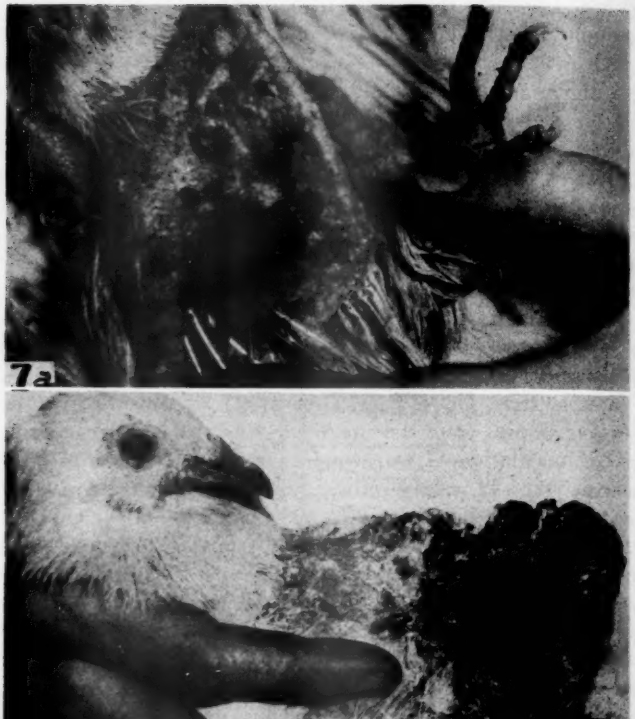
reached at least 18 months, as if the age factor had attained its maximum inhibiting effect at the 3rd month of life.

Both with the tumors temporarily or indefinitely transplantable, numerous tests were carried out trying to find out whether cell free preparations of the growths would induce either non-neoplastic lesions, no matter how mild, or neoplastic lesions in chickens of varying age. Results have been consistently negative despite combining the inoculation of the tumor materials with treatment of the host with a variety of means — cortisone, x-rays, etc. — which one would assume would favor the activity of the hypothetical viruses by depressing the host resistance.

Essentially the same results were observed in sarcomas induced in pigeons, also by m.c., as attempts to show a virus in the latter tumors ended in failure (Duran-Reynals *et al.*, 1945).

In other tests, Dr. J. F. McCrea and I attempted to recover causative viruses from the chemically induced tumors in chickens by inoculation of extracts in the chorioallantois of chick embryos. We did indeed recover viruses, and quite frequently, that can be kept indefinitely by passages through the chorioallantois of chick embryos as well as by passages through chick embryo cell cultures of the Maitland type. Such viruses grow rather slowly in the chorioallantois where they induce lesions similar to those induced by the Rous virus, in the early stages of infection, but which are also similar to lesions induced by other viruses, such as those of the pox group. At any rate, all attempts to induce tumors, or for that matter any sort of lesions in chickens of any age with these viruses, ended in failure, and as of today we do not know the nature of these agents.

We are still working on the subject, as we believe the case of the chemically induced tumors in chickens to be indeed unique. We know that practically every chicken reared under ordinary conditions is or has been infected by a neoplastic virus, as shown by the presence of specific antibodies against the virus of the Rous sarcoma (Duran-Reynals *et al.*, 1953); and we know that most of these chickens will develop a sarcoma under the effect of a chemical carcinogen. It is indeed inviting to try to establish an analogy between these facts and the phenomena, in bacteria, attending the change of a prophage into an infectious phage under the effect of inducing agents, or between comparable phenomena in some animal viruses.



FIGS. 7A and 7B. Two views of a carcinoma induced in a pigeon painted in the skin with a methylcholanthrene solution.

Fowl Pox Infection of Chickens

As said before, if malignancy by chemical carcinogens is mediated by viruses, the first effect of a solution containing the chemical should be to revive the virus from a latent to an infectious state, and logically this virus should be recovered before detectable neoplasia develops. That a given solution of a carcinogen, namely m.c., can revive a latent virus in chickens has been shown in past experiments (Duran-Reynals and Bryan, 1952), the essence of which is as follows: In chickens of the Hallcross

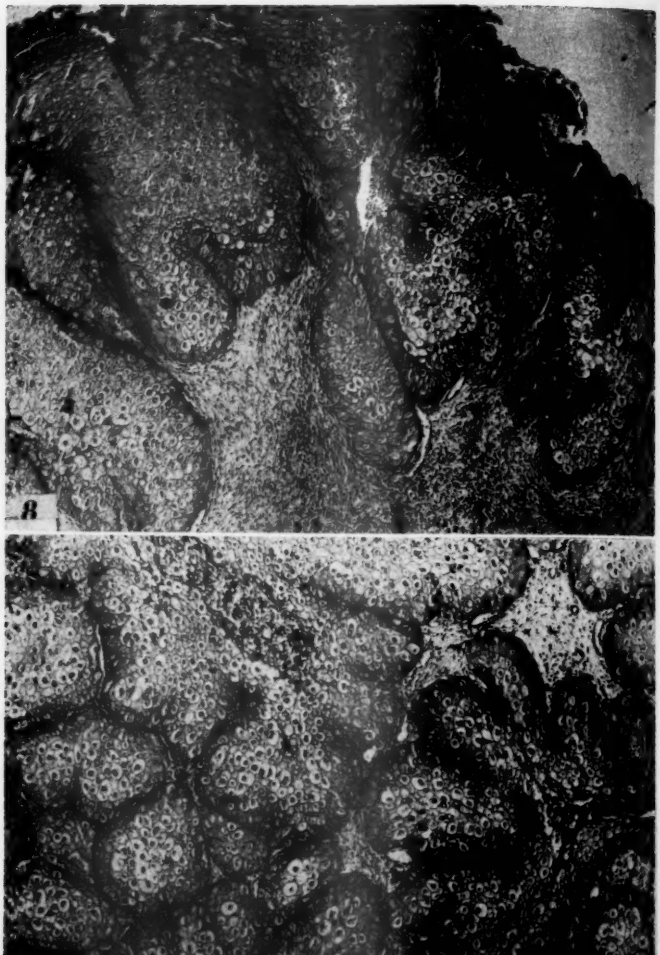
breed, skin paintings with m.c., dissolved in a mixture of mineral oil and ether, result first in the revival of a latent fowl pox infection and later in the appearance of dermal neoplasms — angiomas, papillomas and cancers.* The birds permit persistence of the virus in these neoplasms for long periods of time — several months — after cessation of the paintings, and when no more virus is recovered further paintings easily bring it back.

From these findings one could suspect that the virus is somewhat instrumental in the carcinogenic process, especially considering that this virus is endowed with a strong power to stimulate cells to multiply, thus justifying the term *contagious epithelioma*, by which fowl pox is also known. However, further findings frankly militate against acceptance of this point of view. For, it was found that in chickens from other breeds — Silvercross and Production Barred — the m.c. solution induced the same neoplasms as in the Hallcross breed, without detectable revival of a latent virus.

Then, for a number of reasons, we decided, E. Bryan and I, to study for how long fowl pox virus intentionally injected would persist in the Silvercross and Production Barred chickens, with the following results: If chickens from these two breeds are infected with the virus *as adults*, the virus persists in their tissues for much shorter periods of time than the virus naturally present in the Hallcross breed, and then again in only a number of the birds infected. However, if the chickens from the Silvercross breed are infected *as embryos* 15 days old by injection of the virus in the chorioallantois, the following was observed: Virus is recovered only for about 2 weeks after hatching but if, 4 months later, the chickens are painted with the m.c. solution, the virus is recovered from every bird and for periods of time almost as long as from the naturally infected Hallcross breed.

These results suggest that embryonic infection may result in a state perhaps reminiscent of that described as of immunological tolerance, whereby the virus, revived by the m.c. solution, remains active for long periods of time. The results also emphasize further the relevance in epidemiology of such an early contamination that, after a long period of latency, can be reactivated to

* Paintings of pigeons with the same solution of m.c. also resulted in the frequent development of papillomas and cancers—very keratinizing—but not of angiomas.



FIGS. 8 and 9. Ten-day old lesions induced in the skin of a chick by fowl pox virus. The virus was recovered from a chicken treated with a solution of methylcholanthrene in mineral oil and ether. X 90.

induce an acute infection when the host has reached maturity. The virus, in these experiments, is an ordinary virus and its reactivation is induced by what appears to be rather drastic means, but similar phenomena take place with neoplastic viruses that, after infection of the immature host, become actively neoplastic in the mature host following a long period of latency; the activation of the virus occurring without experimental provocation, as in the adaptation of the Rous virus to foreign species of birds (Duran-Reynals, 1942); the development of avian lymphomatosis under natural conditions (Burmester, 1957) and the experimental infection of certain strains of mice by leukemia virus from other strains (Gross, 1957).

These results, however, fail to throw any light as to whether this fowl pox infection is in any way instrumental in the development later on of the neoplastic lesions. Further experiments to be carried out are indeed required in the attempt to elucidate several points including the part the solvent of the m.c. could possibly have in the revival of the virus.

In what concerns the development of the neoplasms in themselves, the experiments have definitely shown that these lesions can be induced in the skin, in birds, by paintings with m.c., a phenomenon not known before, and confirmed by several workers.

Papillomas in Rabbits

It is in rabbit papillomatosis where the best, perhaps the only clear cut, example of malignancy induced by the joint effects of a virus and a chemical carcinogen is to be found. We are referring to the well known experiment (Rous and Kidd, 1938) in which the papilloma virus injected intravenously induces at once malignant changes in a skin region prepared by paintings with a chemical carcinogen. Since it is known that, if these papillomas are allowed to grow for many months they will become malignant by themselves, we can see that m.c. has induced changes in the tissues that allow this transformation to take place in a very short period of time.

However, the virus in these experiments is a neoplastic virus, and one which, as far as we know, always behaves as such. We tried, in the past, to repeat the experiment of Rous and Kidd with an ordinary virus, vaccinia. The preliminary results were en-

couraging: a number of New Zealand rabbits painted in the flanks with a solution of m.c. and injected intravenously with vaccinia virus developed more papillomas and cancers and at a much faster rate than controls treated only with the carcinogen. However, the results could not be repeated even though, in numerous experiments, we changed the length of treatment with the carcinogen, the strains of vaccinia injected, the route of inoculation of the virus, and still other factors.

A second study is now being carried out also with skin neoplasms induced in rabbits by m.c. treatment. The animals were given 82 paintings in the flank in 3 months. This resulted in the development of papillomas and even cancers, which attained their maximum size 4 months after the end of the paintings, to regress after that period. When regression was well advanced — after another 5 months — the animals were injected with 120 mg. of cortisone in 3 weeks. This resulted in a rapid flaring up of the lesions which attained, at the end of the cortisone treatment, the size they had at their maximal phase of growth.*

The possible presence of viruses in these dermal neoplasms, before and after cortisone treatment, was investigated by inoculating antibiotic treated, bacteria-free extracts of the lesions into the skin of rabbits. So far, the results have been as follows: In actually dozens of tests the extracts secured before cortisone treatment were entirely inactive; however, after treatment with the hormone, the extracts frequently induce lesions in the rabbit skin. These lesions are discrete, largely inflammatory, which both grossly and microscopically are reminiscent of the lesions induced by a mild pox virus, *e.g.*, dermo vaccine, including an initial phase of epithelial stimulation. So far, it has been impossible to maintain these agents beyond a second passage in the rabbit skin, in cell cultures and in the membrane of chick embryos, and consequently no conclusion can be made as to their nature. Experiments on the topic are being continued.

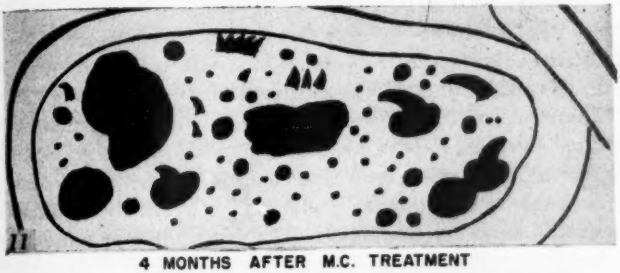
* These experiments have to be compared to those reported by others in mice, in which cortisone has been reported as having either an enhancing or a suppressing effect on the skin neoplasms induced by m.c. (Engelbreth-Holm and Asboe-Hansen, 1953) (Gillman *et al.*, 1955) (Spain *et al.*, 1956). These papers contain references to work by others on the subject.

FIGS. 10, 11, 12, 13. Effect of cortisone on regressing methylcholanthrene induced papillomas of the rabbit. The figure is self-explanatory.

53(B) RABBIT 4652

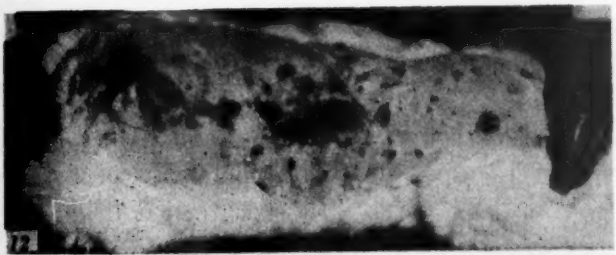
10

1 MONTH AFTER M.C. TREATMENT (82 PTS. IN 3 MO.)



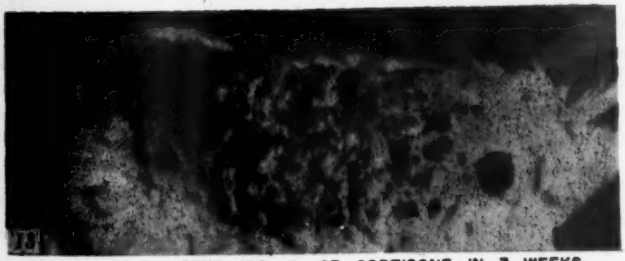
11

4 MONTHS AFTER M.C. TREATMENT



12

9 MO. AFTER M.C. TREATMENT IMMEDIATELY BEFORE CORTISONE



13

IMMEDIATELY AFTER 120 mg. OF CORTISONE IN 3 WEEKS

In the experiments so far reviewed, we must admit that we fell short of our main purpose of demonstrating that infection by viruses — ordinary or neoplastic — is instrumental in carcinogenesis by m.c. Nevertheless, among others, the following interesting facts were disclosed:

(a) that the revival of a latent virus, fowl pox, can be induced in the skin of chickens and of pigeons by paintings with solutions of m.c.;

(b) that the intravenous injection of vaccinia in rabbits painted in the skin with m.c. results, in some cases, in a larger number of neoplastic lesions than that obtained with m.c. only and in a shorter time;

(c) that cortisone induces the flaring up of regressing m.c.-induced neoplasms in the rabbit skin, with concomitant revival of unidentified infectious agents, presumably of viral nature.

Therefore, of the three different experimental models in two animal species, as above itemized, m.c. was a factor in all; pox viruses were a factor in two; and cortisone in one. It was obviously desirable to find another model system in which the three factors could be easily combined, and this system was found in the ordinary, non inbred female Swiss mouse.

Vaccinia Infection in Mice

Let us first give the background to the experiments. It is known that the ground substance of the mesenchyme is conditioned by hormonal influences, as it will increase or decrease depending both on the particular hormone and on the animal species. We found that 100 gamma or less of estrone (estradiol benzoate) injected in non inbred Swiss mice promotes a rapid increase of dermal ground substance manifested by rapid thickening of the skin of the flanks, while cortisone — 5 mg. or less — exerts the opposite effect, with considerable thinning of the skin. These rapid changes occurring in such a common species, as the ordinary Swiss mouse, permitted extensive experimentation on the part played by the ground substance in phenomena of natural and acquired immunity or resistance to virus, bacteria, cancer cells and still other agents; and one of the agents first studied was vaccinia (Duran-Reynals, 1957A).

We found that mice are naturally very resistant to this virus injected intradermally, as the inoculation of 1 to 10 million

minimal doses infective for the rabbit skin of the very virulent Levaditi neuro virus, grown in the rabbit testicle, induces a lesion of but a few millimeters in diameter in the mouse. The lesions are still smaller, or even unnoticeable, if the virus is injected into the very thick skin of mice previously treated with estrone. The situation, however, is entirely the opposite in mice treated with cortisone, and whose skin has therefore thinned considerably. In these animals, the virus induces rapidly a lesion measuring from 1.0 to 1.5 cm. in diameter, which does not heal entirely until 2 weeks later (see Duran-Reynals, 1957B, for illustrations of these phenomena).

These marked and easily reproducible effects of either estrone or cortisone on acute vaccinia infection, in a host so susceptible to spontaneous cancer, as well as to malignancies induced by chemical carcinogens or by hormones, decided us to try again whether the virus infection in a host treated with m.c. and either hormone may not influence the development of neoplasia in a way different from what can be expected from treatment by the chemical, the hormone, or both factors, without the virus.

Accordingly, the mice were painted in the back and both flanks 10 times in 12 days with a 1 per cent solution of m.c. in benzol; 3 days after the last painting the animals were injected, subcutaneously in the groin, some with 100 gamma of estrone in a single dose, and some with 5 mg. of cortisone in daily doses of 1 mg. The virus was injected intradermally 5 days after the estrone inoculation or between the 3rd and 4th injection of cortisone. Naturally, the virus was also injected into other groups of mice prepared in different ways as will be seen later. Grossly, treatment with m.c. does not modify the vaccine lesions in normal mice; nor does it change the inhibiting effect of estrone or the enhancing effect of cortisone on the virus infection.

The microscopic study of these lesions, however, disclosed a marked difference between those in the untreated, as well as in the mice treated only with cortisone, and those in the mice treated with m.c. and cortisone. Although cortisone, as said before, enhances considerably the infection induced by the virus, histologically the lesions are no different from the very mild ones induced in untreated mice. In both groups of animals the lesions are preponderantly inflammatory, with infiltration and necrosis following an initial, short lived, epithelial stimulation. In short,

cortisone alone does not alter the character of the lesions, typical of any dermal infection by a pox virus. In the mice treated with cortisone and m.c. the lesions, grossly similar to those occurring in the host treated with cortisone alone, that is very severe, microscopically reveal, moreover, such an enhancement of the epithelial proliferation as to give sometimes an impression of early malignancy. Methylcholanthrene alone also increases the epithelial proliferation, although it does not enhance the virus infection. Whether this proliferation is as marked as in the mice treated with both m.c. and cortisone is still to be determined.

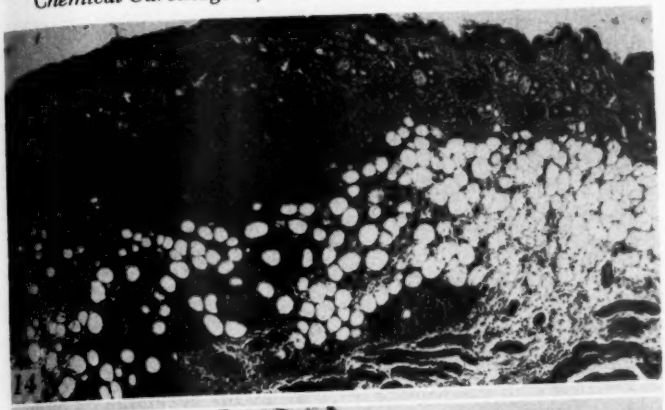
However, these newly formed epithelial cells eventually undergo necrosis and the lesions will heal, although a large scar will persist both in the mice treated with m.c. and cortisone and with cortisone alone.

Now that we know what the virus rapidly, although temporarily, has done to the skin before healing and scar formation, we may be better prepared to understand phenomena taking place in the site of the scar, in the mice prepared with m.c. and cortisone, and *only in these mice*. Anywhere from 3 weeks to several months after the virus infection, and strictly limited to the site of the scar, signs of growth become manifest.

Sometimes they are small, mild, fibrous or papillomatous formations which often regress; but, frequently, these initially mild lesions continue to grow until they become epidermoid carcinomas or sarcomas. We call these growths *site tumors* to indicate that they arise from the site of the virus lesion, whereas we call *random tumors* those that develop in the painted skin but in locations distant from the area of the virus lesion. Some quantitative data have already been given in another publication (Duran-Reynals, 1957b). In a total of 42 mice treated with m.c. and cortisone there occurred 66 per cent of *site tumors*, benign or malignant, and 19 per cent of *random tumors*. The number of mice today available is much higher, and the incidence of *site* and *random* tumors seems to be of the same order.

On the other hand, tumors at the site of the virus injection

FIGS. 14, 15, 16. Three views of acute vaccinial lesions—4 days after virus inoculation—in mice either untreated or treated with cortisone. Note the moderate epithelial stimulation, followed by necrosis, and the marked infiltration of the chorion. Magnification X 50, 175 and 175.



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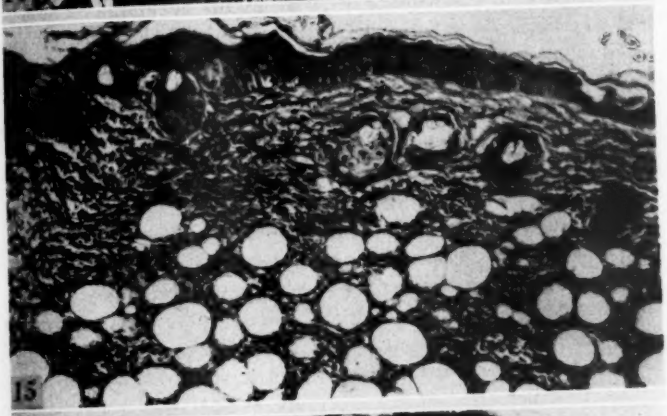

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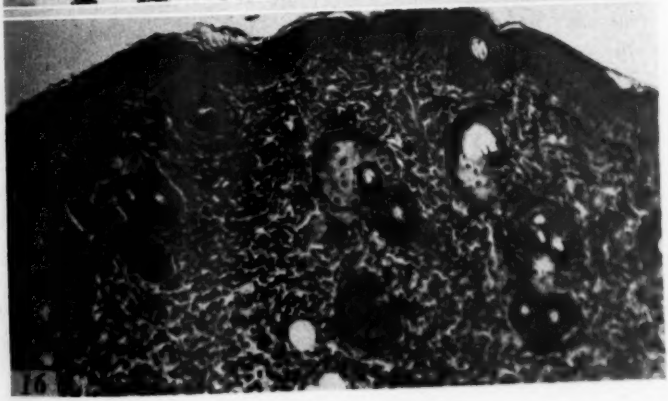

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have never been observed in a total of about 500 Swiss mice variously treated with m.c alone, m.c. plus estrone, estrone alone, or with benzol paintings, cortisone alone or with benzol paintings, and of course untreated mice.

While more experiments are under way with Swiss mice, studies have also been started on inbred strains of mice, DBA/1, DBA/2, C57 brown, C57 black, A, C, and C3H, all from the Jackson Memorial Laboratory. The following has already been learned: untreated female mice from all these strains are as resistant to vaccine virus, or more so, than Swiss mice and, as in the latter strain, the infection is very much enhanced by cortisone treatment. Contrasting with this uniformity of response to the virus, the strains differ very much in their response to m.c. alone, that is in the absence of virus, by the development of dermal papillomas and cancers following 10 m.c. paintings. At the present stage of the study strains A, C3H and C57 black and brown appear to be the most sensitive to the carcinogen.

Tumors at the site of injection of vaccine virus have so far clearly developed, in mice treated with m.c. and cortisone, in strains C57 black and C3H, both very sensitive to the carcinogen, but not in mice from strains C57 brown and A equally sensitive to it. These observations may pose problems of relevance on the importance of the genetic factor as influencing the incidence of random neoplasia in the painted area and the neoplasia developing in the site of the virus lesion.

Another preliminary observation of interest is the following: Mice of the DBA/1 strain treated with cortisone — with or without m.c. — and injected intradermally with the virus showed the customary enhanced lesions, but in addition many of the mice died in 1 to 2 weeks with clean cut large nodules, mostly in the lungs; microscopically these were acute, largely inflammatory structures from which vaccinia was recovered. The lungs consequently get the impact of the infection practically as much, and at the same time, as the skin in which the virus is injected. Generalization of vaccinia is, of course, an ordinary occurrence in

FIGS. 17, 18, 19. Three views of acute vaccinial lesions—5 days after virus inoculation—in mice treated either with methylcholanthrene alone or with methylcholanthrene and cortisone. Note the pronounced stimulation of the epithelia, with incipient necrosis in some cases, together with infiltraton of the chorion. Magnification X 50, 50 and 175.

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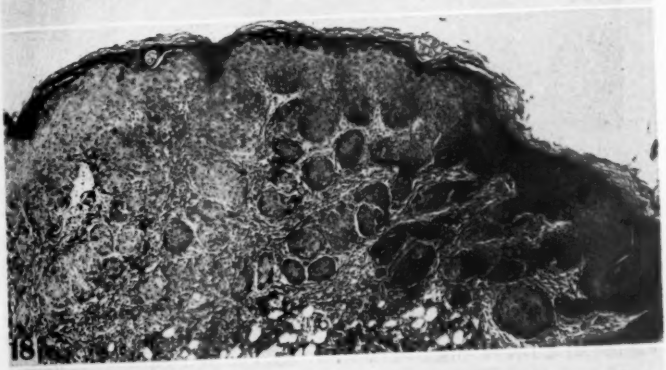

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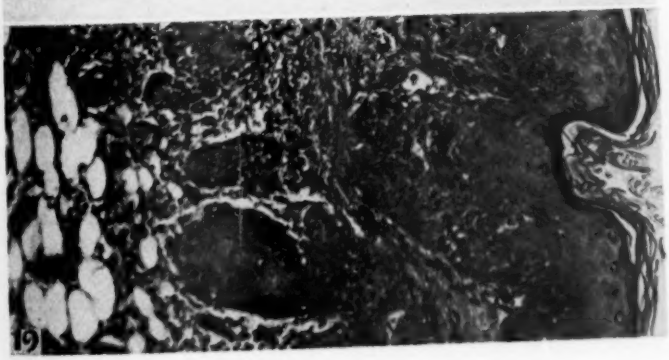

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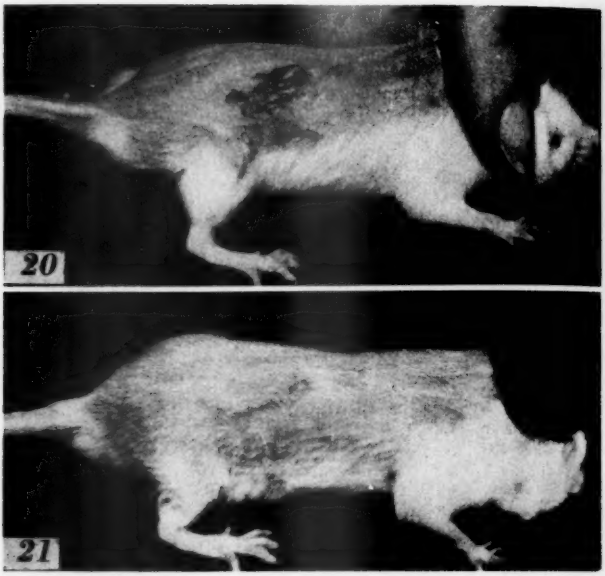

FIG. 20. Vaccine lesion 7 days after virus injection in a mouse treated with methylcholanthrene and cortisone.

FIG. 21. The same 26 days after virus injection. Note clean cut scar, and a minute growth in its upper corner. This lesion grew to attain a size of 3 mm, and regressed later.

hosts, like man and the rabbit, that are very susceptible to the virus. In DBA/1 mice cortisone induces generalization and, if anything, in a more acute as well as in a more selective way, in a host that is normally resistant to the virus. Mice of the DBA/1 strain may turn out to be excellent models in which to study carcinogenesis in the lungs — and perhaps other viscera —similar to that occurring in the skin of mice from the Swiss and other strains.

In summary, in mice from the ordinary Swiss strain, and also from some inbred strains, painted in the skin with an m.c. solution and injected with cortisone, neoplasia often developed in the precise skin site injected with vaccine virus.

Many more data are needed before attempting to reach conclusions as to the intimate nature of the phenomenon. One may be inclined to consider the viral infection as a factor acting like

the co-carcinogens or promoting agents which are supposed to make actual a potential malignant change initiated by the carcinogen. This may be so; but one must admit that our knowledge of the *promoting* effect is probably as obscure as that of the *initiating* effect itself. On the other hand, one may be tempted to envisage the possibility that a non infective component of the virus is involved in the development of the neoplasms.

In another publication (Duran-Reynals, 1957b) we summarized the main studies that are being or will be undertaken at-

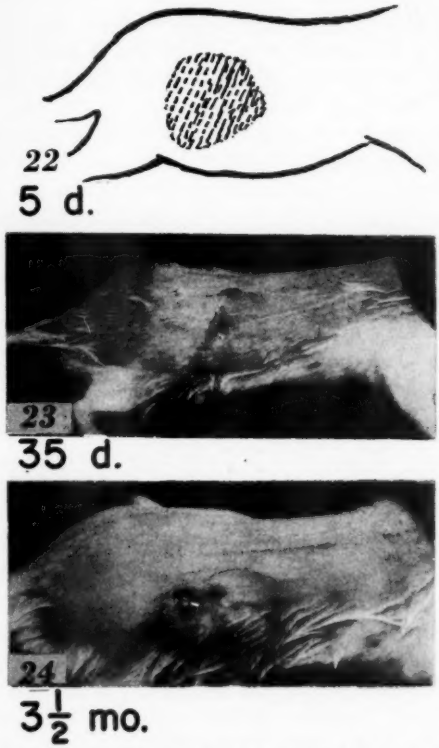

FIG. 22. Vaccine lesions 5 days after virus injection in a mouse treated with methylcholanthrene and cortisone.
FIG. 23. The same 35 days after virus injection. Note scar.
FIG. 24. Sarcoma that developed in the scar 3½ months after virus injection.

tempting to prove or disprove the above interpretations.* We want to add that, in an effort to understand the "promoting action," special attention is being paid to the effect of croton oil, by developing studies already started. Also, tests are being carried out in an attempt to understand the intriguing effect of cortisone, whether it acts by depressing the host reactions against the virus, against the neoplastic cells, or by some other mechanism.

On the other hand, we plan to repeat on rabbits the tests of vaccinial infection under conditions as close as possible to those prevailing in the experiments on mice. This will be done by treating the rabbits with cortisone, besides m.c., and by reducing their extreme susceptibility to vaccinia by a previous infection. Comparable experiments are also contemplated with fowl pox virus in chickens.

Before finishing, we would like to compare our results with those published recently (Wyburn-Mason, 1955) on the development of cancer in humans from the sites of herpes zoster infection, anywhere from 3 months to 3 years after the infection. In this case the sequence of varicella — herpes zoster — cancer is certainly strongly inviting to speculation. One must also quote the compilation of the admittedly rare cases where cancer has developed in the scars of small pox vaccination or in the regional lymph nodes (Helman, 1957).

Summary

The theoretical reasons are given that justify as working hypotheses, first, that neoplasia by chemicals and hormones is mediated by viruses, and second, that, in some cases at least, these viruses may be of the ordinary, inflammatory and necrotizing type which, under certain circumstances, may change into or function as neoplastic viruses.

Experiments attempting to prove the above points are described concerning the following neoplasms induced by m.c.: (a) sarcomas in the muscles of chickens and pigeons; (b) angiomas, papillomas and cancers in the skin of chickens and pigeons; and (c) papillomas and cancers in the skin of rabbits. These experi-

* Let us point out that included in this plan of studies are experiments—now under way—to eliminate the possibility, admittedly very unlikely, that the neoplasms are due to agents, viral or otherwise, but different from vaccinia, which may be present in the infective inoculum.

ments have, so far, fallen short of their main purpose, but a number of relevant observations were made to the effect that: (a) unidentified viruses were recovered from the chicken sarcomas; (b) skin paintings with m.c. dissolved in mineral oil and ether revived a latent infection of fowl pox, in a certain breed of chickens; this fowl pox virus could be recovered from subsequently developing neoplasms for long periods of time and, when recovery of the virus had ceased, further paintings with the m.c. solution consistently brought it back. In another breed of chickens, when the m.c. solution failed to revive a latent infection, the phenomenon can be reproduced by experimental infection of the embryos with fowl pox; (c) cortisone has a strong effect in promoting the growth of regressing skin neoplasms of rabbits and from these neoplasms agents — probably viruses — infectious for the rabbit skin could be recovered, but only after treatment with the hormone; and (d) in rabbits painted in the skin with m.c. — but without cortisone treatment — the intravenous injection of vaccine virus was followed, although only in exceptional cases, by the development of neoplasms, which conceivably could be ascribed to the viral infection.

These facts, together with results from a separate study on the hormonal control of the ground substance of the mesenchyme, motivated experiments on the intradermal vaccinial infection of Swiss mice prepared by m.c. and cortisone. Results showed that after the lesions — much enhanced by the effect of cortisone — have healed, benign and malignant neoplasms frequently developed in the precise site of the virus lesion and, in most cases, in no other location. These neoplasms never appeared in mice treated with m.c. alone, cortisone alone, and with m.c. and estrone, let alone untreated mice. These findings are briefly discussed and plans for future work are outlined.

Dr. Beard: Dr. Francisco Duran-Reynals has been engaged for many years in the study of the relationship of viruses to the occurrence of cancer. He has taken a very fundamental attitude toward this problem, and has contributed enormously to the field as a whole. His paper is now open for discussion.

Dr. Furth, Stanford: I am wondering whether or not carcinogenic hydrocarbons of cortisones is fairly active as a surface agent

with sensory changing the cell penetrability or the cell surfaces for the virus penetration?

Dr. Duran-Reynals: I cannot answer that question.

Dr. Trenton, Houston: I would like to ask Dr. Duran-Reynals if he has attempted to recover vaccinia virus from these tumors?

Dr. Duran-Reynals: You can't recover vaccinia virus from these tumors. The virus disappears two weeks after injection of the virus in the skin of the mice prepared with methylcholanthrene and cortisone.

Dr. Peyton: I would like to indicate the dilemma in which I was in before Dr. Duran-Reynals began to speak. The main issue that he has raised is whether or not a virus remaining dormant in the cell can then be in itself, upon the cell being excited by carcinogenic agent be itself responsible for the production of a fast-growing malignant tumor, or whether the carcinogenic agent produces the tumor, and then the virus merely now multiplies in it in a way in which it couldn't be formed. It was not until I saw this table in which Dr. Duran-Reynals showed the incidence of tumors in mice, with and without previous virus experience, that I think the first real evidence of the causative association of the dormant virus itself became apparent. I wonder whether Dr. Duran-Reynals himself feels that there in that table is the evidence that the virus not merely multiplies in a tumor induced by carcinogenic agent, but that the residual virus in itself caused the proliferation of the cell. Does that table have the crucial evidence?

Dr. Duran-Reynals: It is rather tempting to agree with you, and maybe more definite conclusions in this respect will be reached after a careful analysis of data, such as those of the table, is carried out.

Dr. Kirschbaum: Dr. Duran-Reynals, if we consider that there are stages of carcinogenesis — initiation, promotion, and then growth — couldn't the following interpretation be made, that you have *initiated* carcinogensis with methylcholanthrene, your non-specific viral infection now *promoted*, just as many non-viral agents might promote; this is not initiation of the neoplasm, but a promotional phase, having been *initiated* by a true carcinogen.

Dr. Duran-Reynals: Taking the experiments I reported at their face value there are, tentatively, two ways of interpreting the results obtained. One would be that some residual, non-infective component of the virus is involved in the initiation of the neoplasms in the ground prepared by methylcholanthrene and cortisone. The other would be that the virus, in some form still to be determined and in concert with cortisone, acts as a factor promoting the malignancy initiated by methylcholanthrene. The division of the malignant process into an initiating and a promoting phase is a very useful concept, but it must not be abused or taken too rigidly. The promoting effect is far from being fully understood; it has elements of selectivity and even specificity depending on the animal species, type of injury inflicted, and still other factors. This is why, in our work, we are paying special attention to the effect of croton oil — the powerful promoting agent for mice — to find out whether it can substitute for or modify the effect of vaccinial infection. Some preliminary experiments in this respect have already been carried out.

Dr. Goodpasture: Mr. Chairman, I am very interested in Dr. Duran-Reynals's reports. But I was particularly interested in his reports concerning fowl pox. I have read his original papers on that, and was very much disappointed that the crucial demonstration, that is the microscopic section of his cancerous lesions was not exhibited, because you can get a lot of curious changes during healing of fowl pox, and while I have no doubts he has controlled this business, I would have like to see it. I wanted to ask him whether or not in these lesions of cancer that result from the fowl pox infection treated with methylcholanthrene there is a higher quantity of fowl pox in the lesion than there is in the blood and other organs, as fowl pox can last for many months in the tissues and in the blood stream following an initial infection. I am interested to know whether he thinks there was an actual increase in the fowl pox virus in the lesion itself, and I also would have been interested to know what he found histologically in this lesion that he showed us.

Dr. Duran-Reynals: In our studies on fowl pox virus recovered from the skin of certain breeds of chickens painted with a solution of methylcholanthrene in mineral oil, whenever the virus

was detected in the skin lesions it was frequently found in the viscera of the chickens.

In the mice treated with methylcholanthrene and cortisone and infected with vaccinia, the virus was recovered from the resulting lesions only for about two weeks. Much more work is contemplated on this point, including a study of the generalization of the virus from the skin lesions to the viscera in mice differently treated. Microscopic study of the carcinomas developing in the site injected with vaccine virus shows them to be either epidermoid carcinomas, rather fibrous and keratinizing, or sarcomas. A detailed histological study of these tumors, as compared to those developing after treatment with methylcholanthrene without virus, is under way. I would be very interested to examine the lesions of healing fowl pox to which Dr. Goodpasture has alluded.

Dr. Beard: In view of the passage of time, I think we had better pause for a second here and thank Dr. Duran-Reynals for his very nice presentation.

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THE ACTION OF VIRUSES ON CELLS

E. A. Evans, Jr.*

There are many reasons for the cancer worker's interest in viruses, but I believe I am correct in saying that his interest in the area of bacterial viruses has been especially stimulated because of the phenomenon of lysogeny. Since this is only one feature of our larger knowledge, at a biochemical level, of the bacterial viruses, my intention is to review briefly the current knowledge with respect to these agents.

Bacterial viruses can be classified in two categories, virulent and temperate, on the basis of their behavior towards suitable bacterial hosts. With the virulent virus, infection is followed immediately by viral replication and cell lysis and the virus-host cell relationship is classified as lytic. With the temperate virus, infection is not followed immediately by viral replication, and the relationship is termed lysogenic. It is convenient to consider our information both with regard to the type of virus as well as the kind of relationship separately.

Coliphage T_2, a member of the "T" series of coliphages acting on *E. coli* strain B, may be regarded as a typical example of the virulent type of bacterial virus. Figure 1 is a diagrammatic representation of this agent, summarizing current views of the various components of the virus and their functions. The particles have a slightly elongated hexagonal head attached to a smaller tail, terminating in a thickened end-structure of the indicated dimensions. Highly purified preparations of the virus can be obtained which are homogeneous in both the ultracentrifuge and electrophoretic apparatus. From a variety of analytical procedures, both physical and biological, it would appear that the particle weight is of the order of 200,000.000. Chemical analysis indicates that about 50 per cent of the nitrogen is present as nucleic acid of the DNA type with the rest being protein. Most virus preparations contain measurable quantities of lipid material (about 2 per cent) but it is uncertain whether this is a contaminant or an integral part of the viral structure.

* Department of Biochemistry, University of Chicago, Chicago, Illinois.

Such viral particles as T_2 do not show demonstrable metabolic activity, do not contain energy "reservoirs," nor require any source of energy to maintain their structure. It appears, therefore, that we are dealing with particles which although enormous in molecular size, are bound together by the usual covalent bonds, as well as by such other types of intra-molecular binding forces as operate, for example, in determining the particular configurations of a biologically active protein molecule.

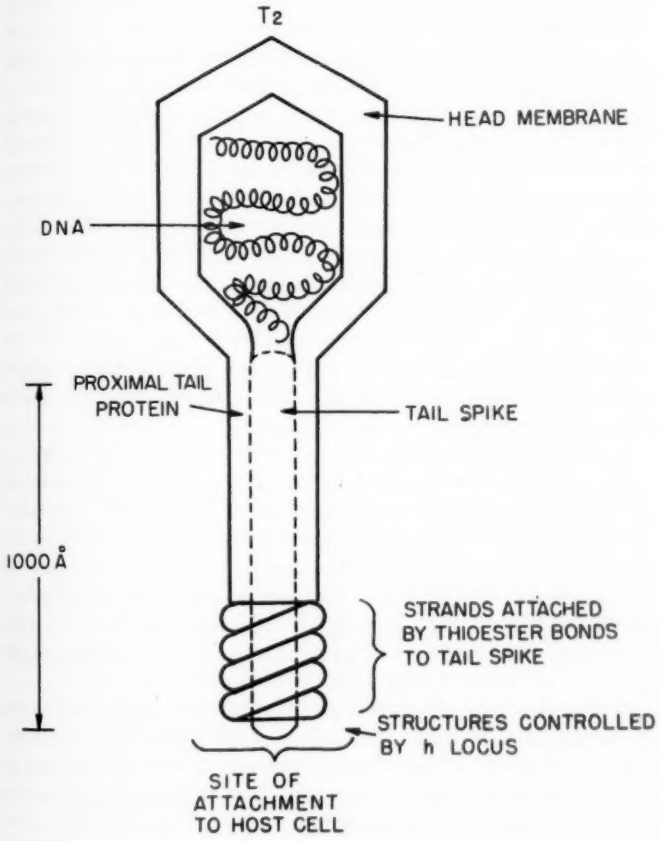

FIG. 1

As indicated in the diagram (Fig. I) it is believed that the nucleic acid of the virus particle is concentrated in the interior of the hexagonal head and is sheathed in a protective coat of protein. Recently Brown and Martin (1955) have been able to separate the DNA from T_2 into two fractions by use of a column of cellulose and calf-thymus histone. The two portions, 30 and 70 per cent respectively of the total nucleic acid, are characterized by differing ratios of purine and pyrimidine bases. Further evidence for the heterogeneity of viral DNA is derived from recent experiments of Levinthal (1955) which I shall describe later.

The most striking feature of the chemistry of the DNA of the T_2 particle is the presence of several anomalous components. Wyatt and Cohen (1953) discovered that the even-numbered T phages contained a new pyrimidine, 5-hydroxy methyl cytosine, which is absent from the nucleic acid of the host cell and appears to replace cytosine entirely in the DNA of this group of phages. Sinsheimer (1955) and independently, Volkin (1954) demonstrated that glucose is also an integral component of T_2 phage, occurring probably in glycosidic combination with the 5-hydroxy methyl group. One is inclined to regard these variations as being of great, if unknown, physiological significance in the function of T_2, but since comparable anomalies have not as yet been found in either the odd-numbered T phages or the temperate phage, it has not yet been proved that they constitute a general pattern in viral composition.

Evidence is also accumulating with respect to the heterogeneity of the protein component of T_2 phage. At the moment, we have evidence for at least four distinguishable protein fractions. The four protein components are: 1) the protein sheath of the hexagonal head; 2) that of the proximal portion of the tail; 3) that in the distal or terminal tail fragment; and 4) that of an internal "spike" or plug-like structure which is only revealed after appropriate treatment of the intact virus particle although the evidence for protein nature here is still insufficient.

Anderson (1953) first showed that the T_2 particle is absorbed to the specific bacterial host by the end structure of the tail. Garen and Puck (1951) demonstrated that the initial phase of the absorption process is reversible, and depends on the existence of complementary electrostatic and geometric rlationships between the virus and receptor areas of the host cell wall. Puck

and Tolmach (1954) were able to show further that with T_2 the initial interaction involves positively charged amino groups—presumably on the protein of the distal portion of the tail—with negatively charged carboxyl groups present on the bacterial cell surface. The first reversible process leads to an irreversible phase which probably involves changes in both the virus particle and the bacterial cell wall. Under normal conditions, this irreversible phase of adsorption is followed by a splitting of the coliphage particle so that the bulk of the nucleic acid of the phage particle passes into the bacterial cell. The protein membrane remains attached to the exterior of the cell and, as shown by the isotope experiments of Hershey and Chase (1952) can be removed without interfering with the further stages of virus reproduction simply by agitating the infected bacterial cells in a Waring blendor.

Further insight into the detailed sequence of events involved in the invasion of the particle has recently been obtained by Kozloff and his co-workers. Their experiments are based on the observation that one can split off quite specifically the tail protein of T_2 by the action of a number of complexes containing divalent Zn^{++} or Ca^{++}. Moreover, the particles obtained by such treatment exhibit greater specific lytic activity on cell membrane preparations from the original bacterial host than does untreated phage material. Also, with mutant strains of the phage that will not attack the host cell membrane, by removing the tail protein by means of the zinc or cadmium complex, one can obtain phage preparations capable of dissolving host membranes (Fig. II, Table 1). It would appear, then, that the role of the tail protein is to function as a specific mechanism for attaching the virus to the host cell, and that its subsequent removal exposes enzymic structures on the proximal portion of the tail which proceed to digest the bacterial membrane (Fig. III). Kozloff, Lute and Henderson (1957) found that the tail protein split off by the action of zinc and cadmium complexes contains about 8 times as much sulfur as the rest of the viral protein. In chemical experiments which I cannot detail here, they have concluded that the splitting off of the tail protein involves the hydrolysis of thiolester groups which serve to knit together the proximal and distal portions of the tail protein. That these observations are related to the *in vivo* process is indicated by recent experiments by Kozloff and Lute (1957).

FIG. II

TABLE 1

Action of Intact and Altered T_2 on Cell Walls of E. Coli Strain B and B/2

Phage Preparation	*E. coli* B (Prep. VII) (Per Cent)	*E. coli* B/2 (Prep. I) (Per Cent)
Intact T_2r^-	4.3	0
Cd $(CN)_4^-$ Treated T_2r^-	8.9	6.7
Frozen-Thawed T_2r^-	7.6	17.0

The zinc content of the bacterial membrane is known to be present at least in part, as a component of a number of dehydrogenase enzymes. When such zinc containing preparations as lactic acid dehydrogenase, alcohol dehydrogenase, glutamic acid dehydrogenase, carboxypeptidase and zinc insulin were added to virus preparations, they were found to be without effect. How-

ever, crystalline glyceraldehyde-3-phosphate dehydrogenase from both yeast and muscle was found to readily inactive T_2. Now the mechanism of action of this enzyme is known to involve the formation and rupture of thiolester bonds. It seems very likely, then, that during bacteriophage invasion, the T_2 tail comes in contact with a bacterial dehydrogenase in the cell wall which contains zinc and which has thiolesterase activity. This enzyme ruptures thiolester bonds in the viral tail, and exposes the viral tail enzyme. This is probably only the first host cell enzyme which the invading virus exploits for its own purpose during replication.

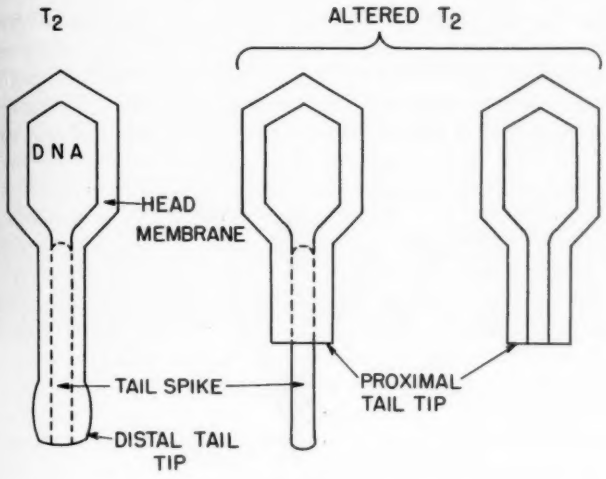

FIG. III

The sequence of events would be those represented diagrammatically in figure IV, involving: 1) interaction between virus and host; 2) splitting off of the distal portion of the tail; 3) lysis of the adjacent areas of the bacterial cell wall; permitting 4) expulsion of the internal spike, together with viral DNA into the bacterial cell proper.

It is not possible to detect the presence of intact virus during the next phase of viral reproduction. If the cells are disrupted during this period, one finds particles which are protein in na-

ture, related serologically to virus protein, but incapable of inducing the virus infection and representing probably intermediate states in viral synthesis. Since the DNA of T_2 contains 5-HMC and is free of cytosine, one can study the synthesis of the nucleic acid portion of the virus by following the changes in phage specific DNA and of host specific DNA. It is found that phage DNA increases some seven minutes after infection while the bacterial DNA decreases. At a time approximately half the period between infection and lysis, mature infectious virus particles begin to appear although the host cell is still intact. Viral DNA continues to increase and remains in excess of that present as infected particles until the cell finally lyses.

The mature virus particle may contain material derived from the parent infecting particle, the bacterial host cell, and the nutrient components of the medium. Since the bulk of the protein membrane of the virus particle remains outside the infecting cell, it is clear that it cannot contribute to the protein of the viral

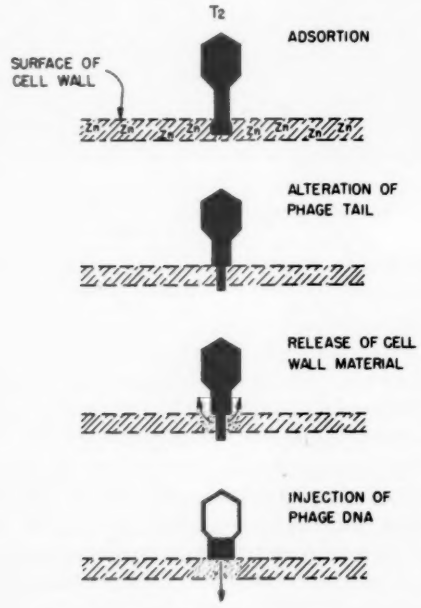

FIG. IV

progeny, and isotopic experiments support this conclusion. The question of the direct utilization of parental DNA for the DNA of the progeny has been a matter of considerable investigation and conjecture. Studies on the fate of labeled viral DNA have shown that immediately after its injection into the host cell a considerable portion can be detected in the acid-soluble material fraction so that some further subdivision of the infected material must be taking place. In earlier investigations, a number of isotopic tracer studies by various workers led to the tentative conclusion that there was no correlation between the transfer of material of DNA from the infecting phage and the transfer of hereditary units, although one can observe the transfer of considerable quantities of isotope from labeled parents to progeny under circumstances in which genetic transfer seems excluded. Levinthal (1955) has recently developed techniques which allow him to measure the radioactivity of a single virus particle labeled with P^{32}, if its atoms emit more than 15 beta particles per month. This involves surrounding the particle under study with a photographic emulsion sufficiently sensitive so that fast electrons leave a visible track which can be observed directly under the microscope. Levinthal's results indicate that the nucleic acid of T_2 consists of one large piece and a number of smaller pieces. The small pieces of DNA are distributed among a large number of progeny virus while the large piece of DNA is transmitted in an intact state to one of the viral progeny. A copy of this essential nuclear core must then be synthesized *de novo* for the remaining virus particles synthesized in the infected cell.

Perhaps the most striking feature of the utilization of the components of the host cell for virus synthesis is the use of practically all of the bacterial DNA. This first involves breakdown of the bacterial nucleic acid into smaller fragments which are then used for synthesis of viral DNA and especially for the viral particles which are formed in the early stages of the infectious process. To the extent that there is sufficient bacterial DNA to account for the whole of the DNA of the viral progeny, relatively little synthesis of viral DNA from the components of the medium takes place. However, as is the case with T_2, when bacterial DNA is insufficient in amount for the DNA of the viral progeny, and when there is necessity for the synthesis of a new component such as 5-HMC (synthesized in part from the cytosine of the

host) then this additional material is synthesized from the simple components of the medium. There is little or no utilization of bacterial protein or amino acids for virus synthesis, and viral protein is formed largely by *de novo* synthesis from the components of the medium.

The picture of virulent coliphage infection which emerges from this is one in which the process of virus synthesis is set off by a fragment of the original infecting particle. The machinery that is used for virus synthesis is for the most part the normal metabolic equipment of the bacterial cell, and the materials on which it operates are the normal components of the bacterial environment and certain portions of the bacterial cellular fabric. Many, if not all, of the normal metabolic functions of the bacterial cell cease, and there begins an immediate and rapid synthesis of the specific parts of the virus particle. Viral DNA appears rapidly. Viral protein can be detected somewhat later, but apparently the two components of the mature particle are assembled only at a terminal stage of the replication process.

When we turn to a consideration of the temperate viruses and of the lysogenic relationship between virus and host, we find much less chemical and biochemical data than is available with their virulent analogues. Little is known about the chemistry of the temperate viruses. Large scale preparations of the temperate virus which lysogenizes the *E. coli* strain K-12 have been made in several laboratories. These preparations appear homogeneous under the electron microscope and consist of tailed particles similar in size and appearance to those of T_1. They contain roughly 50% DNA and are free of 5-HMC.

When a bacterial cell is exposed to a temperate phage, a variety of interactions may take place. The bacteria may be nonresponsive and remain entirely uninfected. Or, infection may occur but the phage disappears in an unknown fashion, *i.e.*, the infection is said to have been aborted, and while the bacterial cell may die, viral reproduction does not occur. Under other circumstances, phage may be reproduced with lysis of the bacterium, *i.e.*, the phage behaves as a virulent virus and the situation is comparable to that described earlier. In the case we are most concerned with, the temperate phage is adsorbed and the bacterium survives to give rise to what is known as a lysogenic clone. The infecting virus particle is transformed (reduced) into what

is termed a prophage, and the host cell continues to grow and reproduce in a normal fashion. In growing cultures of such cells, one finds that in a very small proportion of the cells the prophage passes spontaneously into what is known as a vegetative phase and mature phage particles are then produced. This results in the lysis of the particular cells concerned, with the liberation into the medium of phage particles identical with the original parent. This occurs spontaneously, to a small extent, and for unknown reasons. However, in the case of certain lysogenic strains, it is possible to cause a practically complete conversion of the prophage by treating the lysogenic cells with one of a variety of so-called inducing agents. These include ultraviolet light, soft x-rays, gamma rays or radioactive cobalt, thiomalic acid, reduced glutathione, ascorbic acid, and other organic compounds. When such substances are applied under the proper conditions, conversion of the prophage to the vegetative phase occurs, followed by practically complete lysis of the culture and liberation of virus. The phage produced under these circumstances is identical with the original infecting particles producing this prophage.

As one might expect, this general pattern can be modified by changes in the variables involved in each phase of the process. Even if the phage is of the necessary genetic strain, it is possible, by changing the temperature at which the cells are maintained, or by altering the multiplicity of the infection, to cause the virus to behave in a virulent rather than temperate fashion. Moreover, reduction to the prophage state may be affected by changes in environmental conditions. Even when lysogenization has been accomplished, this can in some cases be reversed by changing the medium in which the material is cultured.

The nature of the conversion of phage to prophage, *i.e.*, the process of reduction, is not known. There is evidence, however, that during the period when reduction is occurring the phage particle behaves as a cytoplasmic unit, while after the lysogenic status has been established, the prophage is firmly associated with or bound to a specific chromosomal site.

At present we have no precise information as to the chemical nature of the prophage. Attempts have been made to look for the specific protein of the infecting phage in a lysogenized strain, but without success, and it appears that viral protein is synthesized only after the lysogenic bacteria are induced. In experi-

ments comparing the fate of DNA from a virulent phage with that from a temperate phage, it has been observed that with the virulent phage a considerable portion of the phosphorus of the viral DNA is found in the acid soluble phosphate fraction of the bacterial cell immediately after infection, with only a portion of the parent DNA being associated with the DNA of the infected cell. With temperate phage, the bulk of the viral phosphorus remains associated with the DNA of the infected cell, at least during the experimental period. On the basis of the very limited experimental data it seems possible that the prophage is either a nucleic acid or a nucleic acid derivative, a hypothesis that is in harmony with the considerable body of genetic evidence supporting the view that prophage is a gene-like unit located at a specific chromosomal site.

The similarities and differences in virus multiplication between induced lysogenic bacteria and those observed after infection of non-lysogenic bacteria have been described in detail elsewhere and cannot be listed here.

In the case of both virulent and temperate infections, the evidence points to reactions involving the genetic material of the cell. One would suspect, therefore, a considerable degree of analogy between the reactions we have described and those which occur in the course of the normal growth and division of the bacterial cell. If the effective portion of the virulent virus takes over and modifies the synthetic reactions leading to the formation of viral DNA, it seems probable that this material must bear a definite resemblance both in structure and function to normal intermediates in cellular metabolism. When one considers the complexity of the process and the product by which viral DNA is covered with its protein coat one might expect again that there should be normal counterparts for such structures. One would like to know something of the structure and cellular location of the normal nucleoproteins of the bacterial cell.

I have yet to mention one aspect of the lysogenization of bacterial cells by a temperate virus which is of the greatest interest. This is the observation that in a number of cases the presence of the temperate virus causes an alteration in the genetic properties of the lysogenized host cell. This will be discussed, I am sure, by Dr. Lederberg in greater detail, and I need mention here only that the transformation from non-toxigenic to toxigenic strains

of bacteria or the ability to synthesize materials that were previously essential nutrients has been observed as the result of lysogenization with temperate viruses.

One can ask, then, what are the general implications of the facts we have considered. It should be emphasized first, however, that the reactions involving the genetic material of the host cell have been occasioned by viruses which contain nucleic acid of the DNA type. While we have as yet no precise and final information concerning the respective functions of DNA and RNA in the normal cell, it is quite clear that these functions, while related, are certainly quite distinct. One would not expect, therefore, that the process of viral invasion and replication involving a DNA-containing virus particle would necessarily be the same for a viral agent in which, like influenza, poliomyelitis, and tobacco mosaic virus, the nucleic acid component is of the RNA type.

Even with such a reservation, the phenomena of lysogeny would appear to be of the greatest interest to the oncologist. We find in lysogeny a process in which viral invasion is not followed by viral replication and destruction of the host, but one in which the presence of the virus is manifested only in terms of changes of the characteristic biological properties of the host cell. If we could equate such changes to those that differentiate the malignant from the normal cell, we would have the basis for an etiology of the malignant process. In other words, the characteristic biological properties of the malignant cell would be the result of the lysogenization of normal cells by a viral agent. This could be formed from other cells by such environmental factors as the endocrines or carcinogenic agents or by the action of such factors on some provirus type of particle already present in the susceptible cell. I must emphasize, in closing, that this is an idea entirely unsupported by experimental evidence. It will be of value when it stimulates investigators to devise experiments that will support or deny such a possibility.

Discussion

Dr. Beard: Ladies and Gentlemen. Dr. Evan's beautiful paper is now open for discussion. This is work of fundamental importance. It's especially meaningful to me because I was one of those short-sighted individuals many years ago who felt that work with

bacteriophage might not be so very productive so far as research in cancer is concerned. But actually the work described here and in the two following papers is of the utmost importance for interpretations and for constituting a basis for going on with the details of the work with the tumor viruses.

Dr. Harlow, Houston: Could you tell me, please, whether the normal *E. coli* has the corresponding enzymatic system to synthesize the large amounts of 5-hydroxymethyl cytosine that are produced in the virus?

Dr. Evans: The question is: Does the host cell contain enzyme systems that will synthesize 5-hydroxymethyl cytosine—not that we know of, the answer is no. There is evidence suggesting that an enzyme system must be synthesized for this purpose after invasion takes place.

Dr. Harlow: That was going to be my reaction that you have a mechanism for the induction of an enzyme system occurring in a very short time.

Dr. Evans: Yes. I have not included all the information about all these systems.

Dr. Haas: You made the statement that with these temperate phages that they did not have any 5-hydroxymethyl cytosine. After they pass from the temperate stage to the virulent stage, what about them? Do they have 5-hydroxymethyl cytosine then?

Dr. Evans: No. The temperate phages can be assayed because we have specific host cells in which they behave in a virulent manner, but they don't change their chemical nature so far as we know, under these circumstances. It would be a matter of great experimental interest to find a temperate virus with a specific DNA label. No one has succeeded yet as far as I know in finding any anomalous aspects for the DNA of the temperate viruses.

Dr. Beard: We shall have to move on to the next paper.

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THE POTENTIAL SIGNIFICANCE OF NUCLEIC ACIDS AND NUCLEOPROTEINS OF SPECIFIC COMPOSITION IN MALIGNANCY

WENDELL M. STANLEY*

The subject which has been assigned to me is the potential significance of nucleic acids and nucleoproteins of specific compostion in malignancy. The work from our laboratory over the years as well as contributions from other laboratories has provided sound experimental evidence that viruses may be composed of nucleoproteins of definite and specific composition. It was shown quite early in our laboratory that tobacco mosaic virus isolated from infected turkish tobacco and from infected tomato plants was identical in all properties examined (Loring and Stanley, 1937). Later the same situation was found to hold in the case of virus isolated from turkish tobacco and phlox plants (Gaw and Stanley, 1947), a finding of special importance because of the fact that the normal proteins of turkish tobacco and phlox show no serological relationship. It is interesting and significant that the same highly complex and specific viral nucleoprotein can be produced in two manufacturing plants, the normal contents of which are serologically unrelated. It has also been demonstrated in our laboratory that different strains of tobacco mosaic virus consist of nucleoproteins which may differ in a reproducible manner in their amino acid composition (Knight and Stanley, 1941; Knight, 1954). For example, ordinary tobacco mosaic virus contains no histidine or methionine whereas the ribgrass strain contains 0.6 per cent histidine and 1.9 per cent methionine. As a result it is easy to distinguish between these two strains in the chemical laboratory merely by making a colorimetric histidine determination. However, despite the fact that viruses do mutate to yield new strains and that growth in different hosts or under different conditions may result in a newly formed mutant strain growing out preferentially and despite the general plasticity of viruses, it must be concluded that a given virus preparation may consist of a nucleoprotein of definite and

* Virus Laboratory, University of California, Berkeley, California.

specific composition. At the same time it must be realized that all virus preparations are somewhat impure in that by the very nature of their production they probably contain greater or lesser amounts of mutants. The actual mass of such mutants in a properly prepared virus sample is probably infinitesimally small, say 0.01 per cent or less, and hence of no real concern from an analytical standpoint.

Previous speakers have shown quite conclusively that some cancers may be caused by viruses, hence it follows directly that nucleoproteins of specific composition have great significance in malignancy and that insofar as nucleoproteins are concerned the word "potential" in the title should be removed. However, it may be well to point out that the significance of this relationship with respect to cancers of man is badly in need of experimental support. Only two little studied and benign virus induced tumors of man are known and viruses have not as yet been seriously implicated in human cancer as generally experienced. This may be due to the fact that few really serious attempts to demonstrate experimentally the presence of a virus in human cancer have been made and to a general reluctance to accept viruses as being of etiological importance in cancers of man. I must say that I continue to be amazed at the willingness of so many investigators to accept viruses as etiological agents for animal cancers and their unwillingness to consider them of potential etiological importance in cancers of man. It should be recognized that cancer is a biological and not a uniquely human problem. Basic biological phenomena generally do not differ strikingly as one goes from one species to another and I regard the fact, now proved beyond contention, that viruses in the form of specific nucleoproteins can cause cancer in animals to be directly pertinent to the human cancer problem. I believe that the time has come when we should assume that viruses are responsible for most, if not for all kinds of cancer and design and execute our experiments accordingly. Cancer is basically a problem in growth and there is no reason to believe the growth of most human cells is different basically from the growth of most animal cells. Acceptance of the viral etiology of human cancer as a working hypothesis will involve a marked change in attitude on the part of many investigators, but this will be necessary if the right approach and the right design of experimentation are to result. What we do in

the laboratory depends in large measure upon what we think. Previous speakers have discussed viral-cancer relationships from a variety of standpoints and I am inclined to believe that the idea that viruses are the etiological agents for most, if not for all, cancer represents a working hypothesis which comes closest to being consistent with all of the presently known experimental facts. Many of the advances which have been described during this symposium now provide for the first time special opportunities to study the possible viral etiology of human cancer. The availability of many kinds of human cells in tissue culture is providing new test, as well as adaptive, systems not only for well known viruses but also for possible cancer viruses, and new methods for concentrating and purifying viruses, and for the removal of inhibitors are providing more powerful techniques for ferreting out possible cancer viruses. We know much more about the interactions between viruses and hormonal, genetic, carcinogenic and age factors than we did only a few years ago. There has never been a time in the past which is the equal of the present with respect to rich opportunities to explore experimentally the possibility that human cancer is wholly or in part due to the activities of viruses.

Following the isolation of tobacco mosaic virus in the form of a crystallizable nucleoprotein (Stanley, 1935; Bawden, Pirie, Bernal, and Fankuchen, 1936), many viruses were characterized as nucleoproteins or as more complex structures having added carbohydrate and lipid and in some cases a complex morphology involving among other things limiting membranes (Stanley, 1947; Bawden, 1950; Luria, 1953). For a long time viruses were regarded as structures at least as complex as a nucleoprotein. Recently, however, my colleague, Fraenkel-Conrat (1956), at the Berkeley Virus Laboratory and almost simultaneously in Germany, Gierer and Schramm (1956) of Tubingen, obtained evidence that nucleic acid obtained by special treatment of tobacco mosaic virus possessed virus activity. This result, astounding at first glance, served to bring into sharp focus the role of nucleic acid in virus activity and, because of my earlier remarks, also the possible role of nucleic acid in malignancy. Actually, the idea that nucleic acid might possess virus activity is not new since genes have sometimes been regarded as nucleic acid and the actions of genes and viruses have long been regarded as being sim-

ilar in quality. Then the transforming principles of the pneumococcus (Avery, MacLeod, and McCarty, 1944) and of *Hemophilus influenzae* (Alexander and Leidy, 1951) have been found to be deoxyribonucleic acids. It may be of some interest that twenty years ago, before the discovery of the chemical nature of the transforming principle, I was inclined to regard this activity as virus-like (Stanley, 1938) for in 1937 I wrote: "It is obvious that there is a factor which may be obtained from any one of the S type of organisms that is noramlly absent from R type cells, but that when added to such cells induces their conversion into the same type of S organisms from which the factor was derived, with the very important result that more of the factor is produced in the induced S cells. This phenomenon is virus-like, and it is because of this and the fact that it may become important from the standpoint of the chemistry of viruses that a discussion is included here. The various type-specific pneumococci may be regarded as cells infected with different 'virus' strains and only the R organisms as healthy. The R organisms may be converted into any one of what we refer to as type-specific organisms by 'infection' with any one of the different 'viruses.' By appropriate treatment it is again possible to free the pneumococci of 'virus' and secure the healthy R type. It is of interest, therefore, to examine the nature of this factor or 'virus'. The type-specificity of the pneumococcus is determined by its capsular polysaccharide, hence it might be assumed that the type of soluble specific substance or polysaccharide isolated by Heidelberger and Avery or the acetyl derivative isolated by Avery and Goebel from pneumococcus type 1 might be responsible for this conversion. However, Dawson, and Sia found that the specific capsular polysaccharide in chemically pure form would not induce the transformation in type. It seems probable, therefore, that, if the polysaccharide plays a role in the transformation, it does so only when in combination with some other substance. Alloway, in attempting to purify the active agent, found that considerable inactive material could be removed by dissolving heat-killed S organisms with sodium desoxycholate, precipitating with cold alcohol, and extracting the precipitate with salt solution. The extract was then heated to 60° C., centrifuged, the supernatant liquid filtered through charcoal, and again precipitated with alcohol. The precipitate was dissolved in water and centri-

fuged to give a colorless, water-clear supernatant liquid containing practically all of the original activity. No chemical tests were made on these purified preparations, hence nothing is known about the nature of the active agent. It is to be hoped that the study of this agent will be continued because of its virus-like nature."

We have come a long way since 1938 and at the present time, of course, the evidence is good that a given transforming activity is due to a highly specific deoxyribonucleic acid (Hotchkiss, 1955). Other characters, such as, for example, sensitivity to penicillin, are now known to be transferred in a similar manner (Hotchkiss, 1951). I still regard this activity, as well as the transmission of hereditary determinants in certain other conditions, as basically virus-like in nature. Needless to say, the demonstration that transforming and related biological activities could be carried by deoxyribonucleic acid represented the first direct experimental implication of nucleic acid in virus or at least virus-like activity. The first inference of a similar nature with an accepted virus was obtained by Markham and Smith (1949) in work with turnip yellow mosaic virus. These investigators isolated a characteristic infectious nucleoprotein from infected plants, but in addition isolated a protein which was non-infectious and yet possessed many properties similar to those of the viral nucleoprotein. It was thought that the non-infectious protein was the protein component of the viral nucleoprotein and hence that the nucleic acid was essential for viral activity. Later on a similar inference became possible as a result of the work of Hershey and Chase (1952) with a coli-bacteriophage labeled with radioactive sulfur and phosphorus. Following adsorption of such phage particles onto susceptible bacteria and separation of the phage ghosts from the infected bacteria the sulfur was found with the phage ghosts whereas the phosphorus was found within the infected bacterial cells. From this it was inferred that the material in the head of the phage, presumably deoxyribonucleic acid, and only this material had been introduced into the bacteria and hence that this material represented the actual infecting element. However, it must be realized that the introduction of some protein was not and still has not been excluded so that this experiment is still not on the same level as that of the transforming principle. This is especially so since all attempts to

separate the deoxyribonucleic acid chemically with retention of activity have failed as yet. Therefore, the discovery of Franekel-Conrat and of Gierer and Schramm that preparations of the nucleic acid of tobacco mosaic virus can carry virus activity is a most important one, since this is the first time that this has been achieved with a regularly accepted virus.

In view of the task which I have accepted to consider the potential significance of nucleic acids in malignancy, I think that it may prove useful to examine this experiment with tobacco mosaic virus and the nature and properties of the biologically active nucleic acid. The first isolation of ribonucleic acid of high molecular weight of about 300,000 from tobacco mosaic virus was made by Cohen and the writer in 1942 by very short heat treatment of a solution of tobacco mosaic virus (Cohen and Stanley, 1942). Unfortunately no tests for biological activity were made on this material. The nucleic acid was found to be highly asymmetric and to decompose spontaneously to form asymmetric particles having a molecular weight of about 60,000. This material could be converted with cold 5 per cent alkali to particles having a molecular weight of about 15,000 and having properties similar to those described earlier by Loring (1939) and others.

Fraenkel-Conrat and Williams (1955) noted that apparently inactive ribonucleic acid of 300,000 molecular weight which had been separated from detergent degraded tobacco mosaic virus could be caused to recombine with inactive native protein obtained by alkali treatment of tobacco mosaic virus and containing no rods 15 by 300 mμ in size, to yield, under suitable conditions, an active virus preparation containing the characteristic rods 15 by 300 mμ in size. For a time it was thought that active virus was being obtained from low molecular weight protein and nucleic acid utterly devoid of virus activity. Fraenkel-Conrat (1956) then conducted experiments in which nucleic acid and protein derived from different virus strains were used and it was found that following infection of susceptible hosts with such mixed viruses the nature of the disease and the chemical composition of preparations of the viral progeny were generally like those of the strain supplying the nucleic acid. This finding indicated that it was the nucleic acid component and not the protein component which was carrying the virus activity. This could be further

substantiated by direct inoculation of the nucleic acid with suitable precautions. All attempts to demonstrate that contaminating rods of tobacco mosaic virus nucleoprotein 15 by 300 mμ in size were responsible for the activity of the nucleic acid failed. One was, therefore, forced to the conclusion that nucleic acid could, in fact, carry virus activity by itself although it seemed that incorporation of this nucleic acid in a protein overcoat was quite desirable for purposes of stabilization and in order to increase its infectivity by about 20 to 100 fold. Gierer and Schramm (1956) obtained similar results with nucleic acid obtained by treatment of tobacco mosaic virus with phenol. In this case, however, the results, which have been confirmed in our laboratory, indicated that the molecular weight of the nucleic acid was around one million instead of the 300,000 or so obtained when detergent was used. It would appear that the use of phenol causes the TMV nucleic acid to come out in essentially one piece whereas detergent causes this unit to break down into about 10 or 12 smaller, yet infectious units. Both groups have found that the virus activity of the nucleic acid preparations, in marked contrast to that of the intact tobacco mosaic virus nucleoprotein rods 15 by 300 mμ in size, was very sensitive to the enzyme ribonuclease and to storage in salt solutions; it was not readily sedimented in the centrifuge and it was not affected by antiserum to tobacco mosaic virus (Fraenkel-Conrat, 1957; Schramm, 1957).

Recently in our laboratory nucleic acid preparations have been obtained possessing potential virus activity as demonstrated by reconstitution with protein equivalent to about one-half of that originally present in the tobacco mosaic virus nucleoprotein used as starting material (Fraenkel-Conrat, 1957). One may, therefore, reach the very important conclusion that the intact virus nucleoprotein rod 15 by 300 mμ in size and 50 million in molecular weight is not absolutely necessary but that all of the stored up information of this structure is actually contained within a ribonucleic acid molecule of around 300,000 molecular weight and hence containing about 1000 nucleotides. This nucleic acid may be prepared sufficiently pure and of sufficiently high biological activity to insure that the activity must be due to nucleic acid structure and not to traces of nucleoprotein or of peptides. The activity may be demonstrated quite readily on direct inoculation of susceptible hosts with the nucleic acid preparation although

because of the instability of the nucleic acid it is usually advantageous to enhance and stabilize the activity by combining the nucleic acid with specific protein. The fact that the nucleic acid may be inoculated directly or following packaging within its own specific but inactive protein or with the inactive protein of related viral strains is providing some very interesting experimental opportunities (Fraenkel-Conrat and Singer, 1957). Now that one can inoculate nucleic acid without its specific protein overcoat, and because the latter is generally regarded as the determinant of host specificity, one can envisage the possibility of infecting hosts formerly regarded as resistant. However, to date infection has been obtained only in hosts known to be susceptible to ordinary tobacco mosaic virus. It is not known whether this means susceptibility or the ability to bring about duplication of the infecting structure is or is not dependent upon the presence within the host cell of a certain pattern of enzymatic activities which, in addition to their synthetic ability, also possess the potential to hydrolyze the specific protein formerly associated with the nucleic acid.

It would now appear necessary to recognize that the nucleic acid structure of around 300,000 molecular weight possesses coded within the arrangement of its 1000 or so nucleotides all of the information which is necessary to bring about in the host cell the production of more of this same nucleic acid, but also apparently the *de novo* synthesis of its own characteristic and highly specific protein with which it eventually coats itself, presumably preparatory to going out into the world. This is, to say the least, a quite wonderful and highly significant course of events. It provides for the first time a direct relationship between specific nucleic acid and specific protein synthesis. Inoculation of tobacco mosaic virus nucleic acid results in the production of the normal TMV nucleoprotein containing no histidine or methionine whereas inoculation of the nucleic acid from the ribgrass strain results in the production of a serologically distinct nucleoprotein containing these two amino acids. The nucleic acid obviously provides the pattern not only for the production of more highly specific nucleic acid but also for the *de novo* production of a highly specific protein. It would be interesting to know the sequence of events within the infected cell since this could throw light on the nature of the synthetic pattern. Are amino acids

first laid down along the nucleic acid in strict accordance with its pattern to yield eventually the polypeptide sequence characteristic of the protein, and, if so, is this done in the first place to yield a polypeptide pattern or template which could then be used to form more of the specific nucleic acid—this sequence of events being brought to a terminal phase by a metabolic situation causing the enfolding of the nucleic acid within a super assembly of the polypeptide chains to yield the final structure consisting of several nucleic acid strands enclosed in a protein overcoat?

It may be of some interest to note that a thousand unit polynucleotide linear chain having the same base composition as TMV nucleic acid could exist in about 10^{590} different arrangements. This number is so large that it is practically incomprehensible. Even a one hundred unit chain of this composition could exist in about 10^{57} different arrangements, and this number is about the same as the total number of electrons on and within the earth and vastly larger than the total of all of the living things on earth. It is obvious that insofar as the possibility of coding information relative to nucleic acid and protein synthesis is concerned, nature has provided a tremendous safety factor if a nucleic acid of molecular weight 300,000 continues to represent the smallest biologically active molecule. Considerations such as those just mentioned might cause one to wonder if considerably smaller nucleic acids possessing virus activity may not eventually be discovered. Truly nature has provided us with some fascinating experimental material, and with patience and intelligence it should prove possible to unravel these interrelationships to the welfare of mankind.

Still another interesting approach is provided by the possibility of packaging viral nucleic acid within specific proteins. So far, it has not proved possible to change the host specificity of a given viral activity by changing the nature of the protein overcoat (Fraenkel-Conrat and Singer, 1957). Experiments in which mixtures of different viral nucleic acids have been packaged within a given protein overcoat have, however, provided some evidence of nucleic acid interaction. Individual lesions produced by inoculation of such material seem to have both genetic components in a higher percentage than when artificial mixtures of the same viral activities in the form of the intact viral nucleoproteins were used. If this result is substantiated in further experi-

ments, it could mean that in a given cell each nucleic acid evokes its own replication independently of other replicating molecules. In one case a new viral strain of intermediate properties was obtained but occasional mutants have also been obtained from a single nucleic acid infection, and there has been insufficient work as yet to warrant the conclusion that the forced intimate association of the two different nucleic acids actually resulted in the production of a new kind of nucleic acid (Fraenkel-Conrat and Singer, 1957). However, it is quite obvious that the packaging of two or more viral strains in the form of nucleic acids within a single protein overcoat must insure that these different nucleic acid molecules enter the same cell and that they should have equal opportunity to initiate the replicative processes. Here again nature has provided wonderful tools with which to explore these very interesting possbilities.

The viral nucleoproteins and nucleic acids I have discussed so far are characterized by their possession of regular virus activity which is readily demonstrable and is not complicated by extraneous factors. We know that many animal and plant tumors are caused by such viruses. However, there are other animal and plant tumors as well as most of the malignancies of man for which no such etiological agent has been found. As I have already indicated I believe that the many new kinds of animal and human cells which are now being grown in tissue culture, as well as the new methods for purifying and concentrating viruses are providing unusual new opportunities, which, when fully exploited experimentally, may result in the demonstration that many of these malignancies of animals, man and plants actually have viruses as their causative agents. Failure to find a virus in a given malignancy need not necessarily mean the absence of a virus for we now have sufficient information to warrant our being ever alert to the possibility of a somewhat different kind of virus activity existing in such cases.

For the purposes of the present discussion I should like to direct your attention to the persistence of a bacterial virus in the form of a prophage in lysogenic strains of bacteria and suggest that this might provide a most significant experimental model for the process that I have in mind (Lwoff, 1952). Lysogenic bacteria perpetuate, in what may be considered a hereditary manner, the property of being able to produce a bacterial virus. The term pro-

phage is used to describe the form in which the potentiality to produce a bacterial virus is perpetuated in lysogenic bacteria. Prophage is non-pathogenic and non-infectious in the normal sense, but since it is multiplied at least once with each cell division, it may be regarded as infectious in the sense that genes or chromosomes are infectious. In other words, the prophage might be considered as a temporary part of the genetic apparatus of the cell and, at the same time, as the non-infectious form of a bacterial virus. When prophage develops into the bacterial virus the bacterium is destroyed, hence a lysogenic bacterium survives only if it does not produce the bacterial viruses. Non-lysogenic bacteria are, of course, well known, and there is at least one well established case in which a strain of lysogenic bacteria, *B. megatherium*, has been converted into a non-lysogenic strain (Gratia, 1936). This fact can be regarded as evidence for the prophage and the resulting bacterial virus being something other than a normal component of the cell.

Of the greatest importance is the fact that treatment of certain lysogenic strains of bacteria with physical and chemical agents, such as x-rays, ultraviolet light, nitrogen mustard, certain reducing agents or iron chelating agents, results, after a latent period, in the lysis of the bacterial cells and the release of large amounts of bacterial virus particles (Lwoff, 1952; Lwoff, Siminovitch, and Kjeldgaard, 1950). These agents are called "inducers" insofar as the production of a bacterial virus is concerned. It is possible that this activation of a prophage by certain and animals. Non-lysogenic bacteria are unaffected by these "inducers" insofar as the production of a bacterial virus is concerned. It is possible that this activation of a prophage by certain chemical or physical agents with development into a fully infectious bacterial virus and the consequent destruction of the bacterial cells provides a biological example of a process which occurs in man? We know that man has coursing through his body many viruses which were unknown a few years ago and we do not know what many of these are doing there (Heubner, 1956). We know that viruses can persist in their host for generations, in either an infectious or a non-infectious form. We know that viruses can mutate to form new strains that cause different disease symptoms. We know that viruses may have different effects depending on the age, genetics and state of nutrition and hormonal balance of the host. We know that different carcinogenic agents

can activate sub-infectious or latent viruses or prophages with consequent cellular destruction. Could it be that the recent discovery of dozens of hitherto unknown viruses of man which seem to be coursing through our bodies from time to time is but the forerunner of the discovery that specific nucleic acids within our cells, perhaps even of exogenous genetic origin, have a relationship to our cancers?

Certainly it would now appear wise to revise the generally accepted definition of a virus to include nucleic acids and perhaps also to include replicating structures which do not evidence infectivity in the usual sense because normally they are duplicated only once or a minimal number of times during each cell division and may never leave the cell during many generations. Such a viral nucleic acid might temporarily appear to be a part of, or associated with, the genetic apparatus of the cell, but be subject to chemical or physical stimulation or shock which could cause it to mature, increase greatly its rate of replication, perhaps mutate, but in any case to separate and act as an independent functional unit. Because of the long history of viruses which indicates they are exogenous in nature and in order to differentiate such a replicating structure from the normal components of the cell, it is imperative that initially the structure or virus must have entered the cell from without. Here the questions of "self" or of "not self" and of "essentiality" are of the utmost importance. Fortunately we now have many powerful devices for characterizing biological entities, hence the demonstration of the existence of a "foreign" or of a "non-essential" component of a cell is far easier than it was a few years ago. Even so Knight (1946) and Beard and associates (Eckert, Sharp, Beard, Green, and Beard, 1955) have found purified preparations of influenza and of myeloblastic leukosis viruses, respectively, to retain antigenic components characteristic of the host in which the virus was grown. In time it may prove possible to separate active nucleic acids from these preparations. I would hope that in order to avoid utter confusion the experimental results will continue to be compatible with the idea that viruses are exogenous in nature even though as has already been indicated we occasionally come very close to the borderline between an exogenous and an endogenous origin. Thus while the reproduction of genetic material from a member of one species in a member of another species might be

accepted, the aberration of a normal component of a given host to yield something which would then reproduce in that host would be incompatible. So far, however, it has not been necessary to trespass for in every case examined carefully the evidence for the virus or self-duplicating mechanism having entered the cell from without at some time in the immediate or far past is good.

In closing may I indicate that I believe that the finding of Fraenkel-Conrat and of Gierer and Schramm that virus activity may reside in a nucleic acid represents a discovery of the first magnitude, for it means that a polynucleotide of comprehendible structural complexity can carry the code or informational pattern not only for the production of more of the same polynucleotide but also for the *de novo* production of a highly specific polypeptide. It provides a much more sound experimental as well as theoretical base for contemplating the existence of the myriads of living things on earth It is a discovery that is affecting the thinking and the course of events in virus research and all that virus research is concerned with, such as genetics, infectious disease, cancer and life itself. Eventually chemists should be able to synthesize a small polynucleotide specifically arranged, hence one may now dare to think of synthesizing in the biochemical laboratory a structure possessing genetic continuity and of all the tremendous implications of such an accomplishment. Studies of this nature and related studies could easily lead directly to the heart of the cancer problem and provide a solution to a situation which today appears so very perplexing.

Discussion

Dr. Beard: It would be presumptious for me to try and comment more than briefly on Dr. Stanley's work and the results he has presented. This is the very heart of the whole problem, and it gives much to go on. Of course, people working on animal viruses, tumor viruses, particularly, have little access to materials now with which to use these thoughts and these concepts and these results. But the time for this is on the way; things are progressing nicely. If these fore-thinking people have provided a basis for the advance of the rest of us turtles, it will be very gratifying indeed. These results are almost unbelievable, and it is a wonderful time to have lived to see developments like these.

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VIRUSES, GENES AND CELLS*

JOSHUA LEDERBERG†

The intimate relationships of viruses and genes have been proper subjects of speculation for many years. More recently, genetic studies, especially on bacteriophages and bacteria have given concrete substance to some of these speculations. For this occasion a brief didactic review of some relevant experimental findings may be appropriate. The support that they can give to concept and methodology in the field of cancer research will be self-evident. More extensive and coherent syntheses of several aspects of virus biology may be found elsewhere (Luria, 1953; Burnet, 1955; Lwoff, 1953; Jacob, 1954; Symposium, 1953; Bertani, 1957). The examples arbitrarily selected here will simply reflect my own experience and interests.

Mutation and Selection

Populations. (The first principle of cellular genetics.) The unit of experimental manipulation is usually a population of individual cells, and these are the units of growth and genetic change. Much confusion has been generated by such statements as "the culture developed resistance to this drug" which contain the hidden assumption that the population has reacted as a unit. Mass responses to external agents are indeed known: these are either nonheritable and physiologically reversible, like enzymic adaptation or cytoplasmic alterations. Heritable adaptation of microbial cultures is usually overgrowth of the population by the fittest cells. However, if the population is not carefully watched, the selective influence may be so subtle, or the overgrowth so rapid, that it will be mistaken for a mass response. A typical example of selective adaptation is the overgrowth of leucine-

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dependent cultures of Neurospora by leucine-independent mutants, in media where leucine is a limiting growth factor (Ryan and Lederberg, 1946). The obvious analogies between neoplasia and growth factor independence were stressed at the time (Lederberg, 1946), but our knowledge of comparative cell nutrition still falls short of the test.

Mutation. Selective action on a population depends on genetic heterogeneity which, in the last analysis, comes from mutation. With higher organisms, the sporadicity of mutation is no longer seriously questioned. Many investigators have, however, hoped that lacking an insulated germ line, microbial heredity might be more readily influenced. Drug-resistance especially seemed to justify this hope in the thinking of many workers. However, a number of examples have been thoroughly studied, and there so far remains no unambiguous demonstration of specific drug-induced mutation, although several claims are still controverted. On the other hand, there are several specific demonstrations of the purely selective action of a drug in the production of resistant populations (Bryson and Szybalski, 1955). This conclusion can hardly be disputed in the face of methods of indirect selection, whereby the resistant clones have never been exposed to the drug (Lederberg and Lederberg, 1952; Cavalli and Lederberg, 1956; Sneath, 1955). Law (1954) has analysed the origin of methopterin-resistant lines of experimental leukemia by related methods, with the same conclusion of a selected change.

The lack of experimental evidence does not, of course, rule out the possibility of the eventual achievement of directed mutation. Rather it speaks for the naivete of our present methods, and the sophistication of the developmental pathways between the genes and their end effects. If, as is now doctrinal, genetic specificity depends on the sequence of nucleotides in DNA, a specific mutagenic reagent should discriminate between different nucleotide arrangements. Nothing short of homologous genetic material is now known to have so nice a preception. However, we may take courage from these bits of knowledge: the second-order specificity of mutagenic and antimutagenic effects, differential inactivation of intracellular components, and the experimental transfer, with functional incorporation, of genetic fragments (transduction).

The point of departure of current studies on chemically in-

duced mutation, mutagenesis, was Auerbach's wartime observations on mustard gases on Drosophila (Auerbach, 1951). Since then, a very wide range of compounds has been studied in bacteria and Neurospora as well (Giles and Lederberg, 1948; Haddow, 1953; Hemmerly and Demerec, 1955; Jensen *et al.*, 1951). These compounds fall into several groups, principally: highly active organic substituting (alkylating) reagents, like the mustards, diazomethane, and formaldehyde; peroxides and oxy-free-radical-yielding reagents like Mn++; and a miscellaneous group, which includes caffeine and other purines, distinguished from the others by low toxicity. The free radicals (OH, OOH) have also been implicated in the mutagenic effects of radiations. By coupling with organic residues, they may also function through alkylation. The mutagenicity of caffeine may be related to natural mutation, because of the occurrence of purines as natural metabolites, and because natural and purine-induced mutation are both depressed by antimutagenic compounds, notably guanosine (Novick and Szilard, 1952).

Like x-rays, the various chemicals are, to a first approximation, indiscriminate in their mutagenic effects.* No single locus is uniquely responsive to any one agent, which therefore will give a wide range of different mutants, usually accompanied by a preponderance of unchanged parental cells. Many genetic loci must relate to indispensable functions; their mutations would therefore be lethal. However, specific local damage probably accounts for only a part of the toxicity of mutagens. The compounds are notable for carcinogenic and carcinolytic properties, and we might wish for more precise information on their toxic mechanisms, not to mention their specificity of attack.

The quantitative mutability of different loci varies in spontaneous mutation and as among different mutagens, notwithstanding their qualitative nonspecificity (Giles, 1951; Demerec *et al.*, 1956). The characteristic reactivity of each locus, irrespective of phenotypic effect, should relate to its chemical or structural configuration. In addition, different agents break

* Some criticisms of the somatic mutation theory of cancer are based on a misappreciation of the random direction of mutagenesis. The same reagents which induce mutations for biochemical defect are also likely to induce reversions to the normal or a new structure. Mutagenesis is in, effect, the momentary excitation of a chromosomal segment to increase the chance of a new configuration. This is, of course, more likely than not to be an ineffective one.

chromosomes at different places (Kihlman, 1952; Haddow, 1953). The task remains of relating cytogenetic to chemical specificity.

Pyrimidine analogues, such as 5-bromouracil, are incorporated into DNA in place of thymine. Twenty per cent of the thymine in *E. coli B* can be replaced without impairing the viability of the cells, (Zamenhof *et al.*, 1956), in contrast to mutation and non-infectivity in substituted phage (Litman and Pardee, 1956). Does the specific replacement in DNA change its function or does bromouracil perturb nucleic acid metabolism more generally (cf. Coughlin and Adelberg, 1956)? Nevertheless this replacement is the most tangible remodelling of DNA structure so far achieved.

Disinfection. Some reagents differentially inactivate plasmids, leaving the remainder of the cell intact. (*Plasmid* is a generic term for extrachromosomal, intracellular, hereditary factors irrespective of their further identification as plasmagenes, viruses, self-reproductive organelles, endosymbionts, etc., *cf.* Lederberg, 1952). This disinfection merges with chemotherapy, in so far as insects can be cured of their endosymbionts by antibiotics—to the detriment of the insect host. Other targets have been the chloroplasts of green plant cells (streptomycin), kappa in Paramecium (chloramphenicol), respiratory granules in yeast and parabasal bodies in trypanosomes (acriflavine), and latent viruses in plants (heat).

Local genetic states. Mutation is thought of as an architectural change in a linear chromosome. Its disorder in time and space has tended to disqualify mutation, and nuclear phenomena generally, as elements of cellular differentation. However, some geneticists are looking to other dimensions of genic change, in the context of "functional states" of the genes. The transposition of heterochromatin or other chromosomal elements can inhibit the action of nearby genes (Lewis, 1950; McClintock, 1956). The change is not permanent, and normal genic function is restored by re-transposition of the inhibitory element to a distant site. The action of a chromosome segment can thus be modified without intrinsic structural perturbations. This furnishes an analogical model for alternation of 'state' in Salmonella phase variation: here the action of a gene for the flagellar proteins switches *on* and *off* without affecting the specificity of the protein made

during the *on* phase. For the sake of discussion, the functional states are distinguished from the numerous stable alleles for antigenic specificity (Lederberg and Iino, 1956). So far, we cannot point to any system where, as we may suppose happens in normal development, the switch is controlled from without. However, the *R* gene of maize is heritably modified by passage through a heterozygote with another allele R^{st}. The modified *R* reverts toward normal state in *RR* homozygotes (Brink, 1956).

Since the transplantation experiments of King and Briggs (1955) point unmistakably to nuclear changes in development, it is just as well that some genetic models are available, rudimentary as they are now.

Viruses and Genes

Recombination. Recombination methodology in bacteria has extended the domain of comparative biology to a group of organisms once thought unique; it has also brought to light virus-gene relationships in a way not yet so practical with other organisms. The classification of bacterial recombination processes is detailed elsewhere (Lederberg, 1955a, 1957); presently relevant are sexuality and transduction, particularly in *Escherichia coli* and *Salmonella.*

Sexuality. As originally described in *E. coli K-12* (Tatum and Lederberg, 1947), sexuality was extremely rare, and selective methods were needed to detect the recombinant clones. For example, two growth-factor-dependent mutants, say a T— (threonine) and an M— (methionine), might be mixed and plated on a selective minimal medium on which only T^+ M^+ recombinants would grow. These could therefore be readily detected, even if they constituted only 10^{-6} of the whole population. Subsequently, other fertile strains, an elaborate compatibility system, and a remarkably fertile "Hfr" strain were discovered (Cavalli, Lederberg and Lederberg, 1953), and the cytological basis of recombination observed (Lederberg, 1956a). This is a side-by-side pairing or conjugation of intact cells which is accompanied by the transfer of a nucleus from one of the cells to the other, and followed by the separation of the conjugants. By mechanically shearing these pairs, Jacob and Wollman (1956a)

and Skaar and Garen (1956) interrupted the matings and limited the genetic contribution which segregated to the progeny*

Transduction. This may be defined as the transfer of a genotypic fragment, in contrast to sexuality which involves an intact nucleus. Like any other classification, this one is arbitrary *e.g.*, a Y-bearing spermatozoon is shy of an intact genome—but the fragments we will have to deal with are quite small, bearing only one or a very few recognizable markers each. The leading example of transduction is Griffith's (1928) pneumococcus transformation. Unfortunately its genetic interpretation was delayed by the sole use of one marker, the capsular polysaccharide, in all the early work. However, Hotchkiss (1951, 1955) showed that resistance to penicillin, resistance to streptomycin, the capsular character, and other markers, are all transferred independently of one another. The active material has been characterized as bits of DNA, with a molecular weight of several million.

From a genetical viewpoint, these bits are broken fragments of the bacterial chromosomes, which penetrating a new host, can resume their original specific functions after replacing homologous chromosome segments. We do not know whether this requires a material ejection, or whether the new fragment reorients chromosome replication (Lederberg, 1955a; Hotchkiss, 1955). The same dilemma, physical breakage *versus* copy choice, pervades modern discussions of crossing-over in higher organisms. It is also unknown whether the bits are formed by random breakage, during the extraction of the DNA, or whether each bit corresponds to a predetermined unit in the living bacterium.

Trandsuction can also be mediated by viruses (Zinder and Lederberg, 1952; Lederberg, 1956b). In *Salmonella*, the phage P22 may be grown on a host, say Gal^+, Xyl^+, M^+ (it really doesn't matter what the symbols stand for, except as genetic qualities, but they are galactose-fermentation, xylose-fermentation, methionine-independence). If this phage is then adsorbed by cells of a complementary genotype, $Gal^-Xyl^-M^-$, these new types can be detected: Gal^+ Xyl^-M^-, $Gal^-Xyl^+M^-$, and Gal^- Xyl^-M^+. That is, a small fraction (about 10^{-5}) of the phage particles carry a Gal^+, or a Xyl^+, or an M^+ gene from the donor

* Certain technicalities on the interpretation of these findings are still under discussion (Lederberg 1955b), but do not affect the utility of the sexual system for genetic analysis.

bacterium. Of course, this experiment is only feasible when the phage leaves a proportion of survivors. The role of the phage in these experiments is merely as a passive carrier of the genes of the donor cell. The transforming abilities of any phage preparation are strictly determined by the character of the cells on which the phage was most recently grown. A number of *Salmonella* phages have been found to act in the same fashion, as has the phage Pl in *E. coli* (Lennox, 1955). Other phages have hardly been examined for this ability, not to mention viruses for other organisms, where technical problems have still to be overcome.

In *Salmonella*, the transduced genetic fragment, or *exogenote*, is readily separated from the phage nucleus that accompanies it within the same coat. When the phage is treated with x-rays or ultra-violet light, its infectivity may be completely destroyed before the transducing activity is impaired. When a cell is mixedly infected with several phages, one of which is carrying a given exogenote, the transformed clone may become lysogenic for any one of the input phages at random (Zinder, 1955). In certain poorly adapted phage-bacterium combinations, most or all of the transformed clones may remain sensitive or immune to the transducing phage. The envelopment of the exogenote within the phage coat makes it inaccessible to direct chemical analysis at the present time, but DNA-transduction is to phage-transduction as a postcard is to a letter: the message is the same.

Prophage-linked transduction. E. coli strain K-12 is lysogenic for a phage called *lambda*. Unlike the generality of the *Salmonella* and pneumococcus systems, the only markers known to be transduced by *lambda* are a cluster of Gal (galactose-fermentation) loci (Morse, Lederberg, and Lederberg, 1956a,b). *Lambda* obtained by ordinary lytic growth is ineffective, and it must be obtained by the UV-induction of lysogenic bacteria. The immediate result of a transduction is often a *heterogenotic* clone, in which the exogenote persists and multiplies side by side with the corresponding homologue from the recipient bacterium. As a further event in these clones, a bacterium may undergo segregation, whereby the exogenote will undergo crossing-over with the chromosome, and give rise to any of a variety of recombinant types. (In other cases of transduction, the heterogenotic phase is evidently very brief.) The peculiarities of this system

are thought to depend on the linkage of *Gal* to the *lambda* prophage, as will be elaborated shortly.

Lysogeny and lysogenic conversion. The fate of a sensitive bacterium infected with *lambda* is indeterminate: it may lyse and release a crop of progeny phage, or it may survive to give a clone with lysogenic and sensitive components. The lysogenic bacterium is one which proliferates normally, or nearly so, but cells of which may lyse from time to time and release phage. The *prophage* has been defined as the genetic element that differentiates a lysogenic from a sensitive cell (Lwoff, 1953). A more general usage, which may not be identical in meaning is that the prophage is the element of a lysogenic bacterium which is regularly incorporated in the phage, *i.e.*, it is the whole phage nucleus as this is represented in the lysogene bacterium; a part may be present in the sensitive cell. Induction or maturation of the prophage into infective, mature phage, which precedes the occasional lysis of a lysogenic bacterium is markedly stimulated by ultraviolet light and many mutagenic chemicals.

The sexual recombination system in *E. coli* K-12 has furnished a unique opportunity to study the genetic basis of lysogenicity. Crosses of lysogenic with sensitive strains, and especially the occurrence of diploids which are heterozygous for the trait, showed that the prophage is fixed at a specific bacterial locus, *Lp*, which is linked to another locus, Gal (Lederberg and Lederberg, 1953). Lp^+ or the presence of the prophage, can be distinguished from Lp^s, the standard sensitive, by two effects: immunity to the infective virus, and productivity of it, especially upon exposure to UV. If it were not for the latter, the alleles Lp^+ Lp^s could not be distinguished from any other genetic markers in their fundamental behavior.

The hallmark of a virus has been thought to be infectivity, or transmission from cell to cell through the medium. The discovery of transduction has largely obliterated this distinction. In fact, it is appropriate to categorize lysogeny as a kind of transduction, not merely *by* phage, but *of* the prophage. In *Lp-Gal* system, the prophage is transduced along with the linked *Gal* marker. They are readily separated: in fact, from unselected donors, only 10^{-5} of the phage particles preserve the coupling. However, the phage from heterogenotes shows an efficiency of transduction for *Gal* which is nearly one. The exogenote here is

derived from one previously selected for the coupling of *Gal* and *Lp* in the phage.

The ordering of lysogeny as a subclass of transduction is more than a verbalism. Lennox (1955) and Jacob (1955) have shown that Lp^+ *i.e.*, lysogeny, can be transduced by *other* phages in linkage with *Gal*. Furthermore, many examples of *lysogenic conversion* have been found in which a bacterial trait is intimately associated with a given prophage which then serves as a transmissible gene for that trait. Apart from immunity phenomena, which are almost inevitable adaptations to the viral life cycle, specific prophages are associated with toxin synthesis in *Corynebacterium diphtheriae* (Freeman, 1951) and with the somatic antigens in *Salmonella* (Iseki and Sakai, 1953; Uetake *et al.*, 1955). In the latter case, the same phage which is responsible for the conversion also transduces casual fragments at a low efficiency. The lysogenic conversion effect is so inherent in the prophage that it is independent of the immediate donor. This formulation suggests that lysogenic conversion is an extreme case of linkage of bacterial markers to prophage, and predicts that further study should show an occasional separation (*cf.* Terada, 1956).

It must be admitted we do not know the precise mode of association between the prophage and the chromosome. By analogy with other transductions it may be a linear segment, peculiarities such as inducibility being an evolutionary specialization. The same evolutionary argument would allow for other geometries, *e.g.*, a lateral attachment. In any case, the phage nucleus in a lysogenic bacterium (or at least *E. coli*) is a specialized part of the bacterial nucleus.

Host-controlled variations. To this point we have been concerned with the genetic effects of viruses on host cells. Converse effects are also important. For example, an elaborate system of host-controlled variations of phage underlies the typing scheme for *Salmonella typhi*. Phage grown on any given type of host becomes specifically adapted to that host, and initially attacks other types very inefficiently. Anderson and Felix (1953) found that the principal determinant of host type is a prophage quite distinct from the Vi_{II} typing phage. These studies therefore exemplify lysogenic conversion and host-controlled variation at the same time. Prophage determinants have, in fact, been implicated

in most examples of modification. Where they have not been found, *e.g.*, in the modifications of lambda (Bertaini and Weigle, 1953; E. Lederberg, 1954) the determinant gene might be a mutant prophage (*cf.* Jacob and Wollman, 1956b; Appleyard, 1956). As already indicated, we have no operational criterion to argue for or against this origin of a bacterial gene.

A number of more or less remote analogies suggest themselves for the biology of host-controlled variation. Genic modifications were mentioned earlier. In *phenotypic mixing*, two viruses actively growing in the same host jointly determine the phenotype of the protein coats of the progeny phage, regardless of the genotype contained in any single coat (Streisinger, 1956). Transduction exemplifies the incidental carriage of other materials from the host. Finally, there may be obligatory recombination between the host and the phage nuclei in some combinations. Radiobiological studies (Garen and Zinder, 1955) independently suggest that a radiosensitive target occurs both in the host cell and in certain phages. This target must be inactivated in both sites to prevent growth of the phage. Mutations have not been induced by treatment of free virus, but they occur when both the bacterial host and the phage are irradiated (Weigle, 1953), further evidence for a regular mechanism of genetic interchange between the two.

To emphasize the intimacy of virus-host relationships, Luria has spoken of 'parasitism at the genetic level'. We now have the means to deal with viruses as 'genes which function at the parasitic level'.

Humility and a sense of a proportion concur in stressing the reaches of our ignorance. For technical reasons, bacteria and bacteriophages occupy an active salient. On one flank, they support the inevitable decipherment of genetics as biochemistry. On the other, they have provoked a methodology by which a cellular genetics can be built on the model of the microbes (*cf.* Puck and Fisher, 1956; Lederberg, 1956c). The very obscurity of this field at least leaves room for optimism. Nature may have some surprises in store for us, if we but look for exploitable processes of genetic interaction. If this fails we can still resort to art, for if nuclei can be transplanted (King and Briggs, 1955) why not chromosomes or their parts? In the long run, the genetic bases of the neoplastic phenotype can only be established with

the tools of direct genetic analysis, a remark which echoes the state of bacterial genetics just fifteen or twenty years ago.

Discussion

Dr. Beard: We have witnessed a most remarkable phenomenon here today. Dr. Sabin has carried out his chore, and he has ended two and a half minutes earlier than he was supposed to. This is really a unique occurrence. It is a little late, but I think it would be bad indeed to pass up the opportunity for the discussion of these very splendid pieces of work. Would someone care to ask questions of Dr. Stanley, or of Dr. Evans, or Dr. Sabin, or to make comments?

Dr. Sabin: Mr. Chairman, I promised to read Dr. Lederberg's paper with the understanding that I would open the discussion of the points he raised. I would like to use the board, if I may. The models which these studies provide for us, lead us to think of the various ways in which viruses might be involved in the production of malignant tumors. We have all heard a great deal about the tumor viruses in the chicken which behave remarkably like ordinary viruses, but they may differ from other tumor viruses only in the sense that they are much more readily liberated from the cell—and instead of being in a prophage state, instead of behaving like lysogenic viruses coexisting with the proliferating cells, they are liberated. But the phenomenon of transduction, I think, really provides a model that needs to be investigated in the field of mammalian tumors—both experimental and human tumors. The hypothesis may be like this—you have a cell here which has a nuclear material that will ultimately turn the cell cancerous. There may be many reasons for the "cancerous" nature of this nuclear material—we are not concerned with that now. This "precancerous" cell exists in an organism that has many exposures to different viruses during the course of its life. The innumerable Echo viruses we carry in our intestinal tract, the innumerable respiratory viruses we carry in our respiratory tract certainly assume possibilities that this ultimately cancerous cell may be invaded by one of the viruses and thus become associated with genetic material that has an ultimately cancerous destiny. Now the question that arises is this: Is it possible that when such a virus then leaves the precancerous cells to infect

another human being, that it may carry the cancerous nucleic acid determinants into normal cells and render them cancerous —the virus being only the "postman"? Now this brings us a little bit closer to the question that Dr. Kirschbaum has raised—namely, that we have to decide somehow between a primary cause and a secondary cause. Could we, under these circumstances, say that a virus which could be shown to transfer cancerous nucleic acid material is a primary cause? It obviously would not be a primary cause because it could not produce cancer without having obtained this cancerous material from a "pre-cancerous" cell. Can this hypothesis be tested with materials currently available in experimental cancer work? I think it can, because we have animals which can be infected by viruses which do not destroy the cells. I have reference here particularly to the strain of mice that I worked on a number of years ago—the "PRI" mice that are genetically so constituted that certain viruses can multiply in them without destroying the cells or destroying the animals. I think there is an opportunity for testing the capacity of a virus like that of Russian Spring-Summer encephalitis with which Dr. Alice Moore has worked. To test the capacity of such a virus after it has multiplied for many generations in experimental cancers grown in "PRI" mice to transfer "cancerous material" to various normal cells. This is only one example of an approach which I think we do have. We can grow other viruses repeatedly in the presence of cancer, and test their capacity to induce cancers experimentally. However, unless we can utilize the tissue cultures and find some means whereby we can recognize a malignant change in a normal cell the work will be greatly limited.

Dr. Bittner: This has been a most interesting morning, but I would like to throw out another suggestion as to the possible action of these infectious agents or viruses. That is, that agents may be acting like enzymes, either directly or indirectly, on the host itself. Now in the case of our agent, we have been thinking in terms that the agent may be altering the hormonal pattern of the host—that is, either directly or indirectly on other enzymes Now we had the idea about ten years ago working with Leo Samuels. We could not confirm that work on the difference in the excretion of 17 keto steroid, because the sample that Dr.

Samuels threw away contained a large amount of these steroids. But one of my associates of the present time, Dr. Franz Harlberg, is getting suggested evidence that there is a difference in the mitotic activity of certain cells in the host. In other words, we are working with the animal instead of the test tube. Mice having the agent have certain cells that divide more rapidly than mice without the agent. These hormones acting on the mammary cells eventually could cause the cells to become cancerous. That might imply that the agent is not the primary causative agent; it works indirectly. It does not mean that the agent is no less important than if it were a cancer virus. We also have evidence that cancer can be induced in the mice without the agent. Dr. Muelbock in Amsterdam has found that if he grafts pituitaries into agent-free mice, in certain groups 100 per cent of his mice will have mammary cancer. Those tumors, as far as he could tell, did not possess the agent. Now there, he has altered the hormonal pattern of the host to produce cancer in the absence of the agent. If the agent works through the hormones, we could visualize mice with the agent as having more "carcinogenic hormones" than mice without the agent. This is another possibility of the action of an agent or a virus in the ideology of cancer, and it is the program which we have underway at the present time. Within maybe five years, we should have some more information.

Dr. Trenton: I would like to ask Dr. Sabin with reference to his stimulating last analogy if he would accept the possibility that his postman happens to be a very particular postman in that he would not pick up just any kind of mail but only specific mail. I have reference to the fact that the milk agent is present in most if not all tissues of the body can be obtained from it and put into susceptible mice not containing the agent, and regardless of the tissue from which it originated, and in many cases in strains of mice that are susceptible to many other tumor types it carries with it only the ability to produce mammary tumors.

Dr. Kirschbaum: Dr. Sabin proposes that the change is essentially genetic. Extrinsic agents affecting large numbers of cells might induce the critical genetic change and then the affected cells reproduce with this alteration. I have reference to radiation or chemicals which might produce the same genetic alteration.

This need not be a virus which enters the cell; multiple agents might induce the same types of alteration in the gene itself.

Dr. Beard: Folks, I think it is about time to bring this meeting to a conclusion. I should like to say for those of us who have been sort of pinned to the mast here for the last few days, that this has been a real pleasure. It's a great town; it's a fine research center; it's been a fine audience, and we have apprciated it. There is one more thing that I should like to say. The make-up of this program and the mole work of this affair has been done by one of your associates, Dr. Dmochowski. I think, in ending this program, that we ought to give him a wild burst of applause for his efforts that obviously have been quite successful.

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CALLING ATTENTION TO

Items of Possible Interest to Our Friends

April, 1957

Preclinical: HA Abramson & Co find beef brain extract blocks lysergic acid diethyl amide reaction in fighting fish (*Science* 125/397/Mch 1/57). AF Brodie & CT Gray suggest bacterial particles (like mitochondria) for studying oxidative phosphorylation (*ibid* p 534/Mch 22/57). M Calvin & PB Sogo discover primary quantum conversion process in photosynthesia (*ibid* p 494/Mch 15/57). H Chiodi details respiratory adaptations to continued high altitude hypoxia, while EL Becker & Co show decreased renal function on high altitude acclimatization (*J Appl Physiol* 10/79, 81/57). S Ellis discusses some metabolic effects of epinephrine & related amines (*Pharmacol Rev* 8/485/56). H Gangloff & M Monnier show locus of action of anticonvulsive drugs (*Electroenceph Clin Neurophysiol* 9/43/57). R Lotmar notes sulfur distribution (most in cartilage) after labelled Na Sulfate administration (*Ztschr Exp Med* 128/243/57). EH Pinson & WL Langham describe physiology & toxicology of tritium, showing skin absorption of HTO (*J Appl Physiol* 10/108/57). H Rahn & WO Fenn give *A Graphical Analysis of Respiratory Gas Exchange* (Amer Physiological Society, Washington, DC, 57, 38pp, $4). EG Rennels differentiates 3 types of gonadotrophic basophils in pituitary (*Ztschr Zellfor mik Anat* 45/464/57). BB Ross describes influence of bronchial structure on lung ventilation (*J Appl Physiol* 10/1/57). HM Rozendaal & Co produce new compounds by radiating l-tryptophan, yielding growth inhibitors (*Science* 125/492/Mch 15/57).

Clinical: WC Alvarez calls attention to abdominal symptoms of little strokes (*Geriatrics* 12/164/57). WH Beierwaltes, PC Johnson & J Solari discuss *Clinical Use of Radioisotopes* (WB Saunders, Philadelphia, 57, 451pp, $11.5). FF Foldes discusses *Muscle Relaxants in Anesthesiology* (CC Thomas, Springfield, Ill, 57, 210pp, $6). MA Gardes & M Lanlan find benzilic acid diethylamino-ethanol ("Benactyzine") useful in control of manic-depressive psychoses in doses of 6 mgm daily (*Presse Med* 65/180/Jan 30/57). GW Granger suggests psychiatric patients have raised intensity thresholds in dark adaptation (*Science* 125/500/Mch 15/57). DF Gray & AJ Ersley find hypoxic serum gives reticulocytosis, suggesting humoral erythropoietic factor as indicated long ago (*Proc Soc Exp Biol Med* 94/283/57). CM Gruber Jr & JM Mosier find 2-p-chlorphenyl-3-methyl-2,3-butanediol (phenaglycodol) effective anticonvulsant in epilepsy associated with focal brain damage (*Proc Soc Exp Biol Med* 94/384/57). FB Murphy discusses status of allergy in connective tissue diseases (*South Med J* 50/57/57). WF & MW Neuman show relation of structure to metabolic functions of bone (*Amer J Med* 22/123/57). AW Oughterson & S Warren edit *Medical Effects of Atomic Bombs in Japan* (McGraw-Hill, NY, 57, 709pp, $12.5). WT Pommerenke well discusses genetic & legal aspects of artificial insemmination (*Obs-Gyn* 9/189/57). RG Skillen

& Co find chylomicron index increased in multiple sclerosis & that pepsin with pancreatin & bile salts lowers it (*Proc Soc Exp Biol Med* 94/300/57). JL Svirbely & BE Saltzman find ozone toxic at 6 ppm with possible tolerance (*Arch Indust Health* 15/111/57).

General: VG Dethier analyses insect communication by dancing (*Science* 125/331/Feb 22/57). R Dugger explains what corrupted Texas (*Harper's* Mch/57, p 68). WA Fowler outlines formation of elements (*Sci Month* 84/84/57). JA Gengerelli takes hard look at dogma & discipline of psychoanalysis (*Sat Rev* Mch 23/57). RB Goldschmid paints pleasant *Portraits from Memory: Recollections of a Zoologist* (Univ Washington Press, Seattle, 57, 181pp, $3.5). LR Hafstad well discusses science, technology & society (*Amer Sci* 45/157/57). R Lasker describes book-devouring silver-fish, the paper eating insects (*Sci Month* 84/123/57). RB MacLeod discusses teleology & theory of human behavior (*Science* 125/479/Mch 15/57). RA McConnell gives surprisingly uncritical account of psi phenomena & extra-sensory perceptive methodology (*Amer Sci* 45/125/57). DC McClelland compares S Freud (1856–1939) & CL Hall (1884–1952) as pioneers in scientific psychology (*Amer Sci* 54/101/57). F Osborn hopefully surveys our reproductive potential (*Science* 125/531/Mch 22/57). SJ Perlman cheerfully charts *The Road to Miltown* (Simon & Schuster, NY, 57, 273pp, $3.5). M Polanyi considers the sickness & cure of scientific outlook (*Science* 125/480/Mch 15/57). PF Schmidt analyses geometrical, physical & logical models of scientific thought (*Amer Sci* 45/137/57). JH Shera & Co edit *Documentation in Action* (Reinhold, NY, 57, 471pp, $10). CH Thigben & HM Cleckley depict *The 3 Faces of Eve* (McGraw-Hill, NY, 57, 320pp, $4.5).

MAY, 1957

Arthur Stoll's 70th Birthday is memorialized by magnificent *Festschrift* of 64 articles by world's best medical chemists, & P Schiff's analysis of his 300 scientific papers (Birkhauser, Basel, 1957, 935pp). For Stoll, H Blaschke describes metabolism & storage of biogenic amines (*Experi* 13/9/Jan 15/57); G Hesse, B Banerjee & H Schildknecht describe mimosa plant irritants as reductones, & H Theorell reviews nature & action of oxidation enzymes (*ibid* 1, 13).

Biological methodology: HJ Antweiler edits *Die quantitative Elektrophorese in der Medizin* (Lange & Springer, Berlin W35, 57, 317pp DM20). RC Mellors edits *Analytical Cytology: Methods for Studying Cellular Form & Function* (McGraw-Hill, NY, 57, 512pp, $12). WS Spector edits *Handbook of Biological Data* (WB Saunders, Phila, 57, 584pp, $8).

Preclinical: GA Emerson offers "representative recent contributions of pharmacologic literature", a fully documented monthly index covering over 100 journals. Federation Amer Soc Exper Biol for April meeting in Chicago brought blizzard of 1888 abstracts (*Fed Proc* 16/1–477/57). P Hochstein says hormonal regulation of glucose phosphorylation occurs in mitochondria (*Science* 125/496/Mch 15/57). SS Kety edits *Pharmacology of Psychomimetic & Psychotherapeutic Drugs* (*Ann NY Acad Sci* 66/417–840/57). PJ Kramer discusses ion accumulation in absorption by active transport against concentration gradient (*Science* 125/633/Apr 5/57). HD Moon & L StVincent

show somatotropin increases rate of protein synthesis & number of cells (*ibid* p 643). IH Page pleasantly resurveys brain chemistry (*ibid* p 721, Apr 19). RE Peterson & Co show amphenone (1,2-p-aminophenyl)$_2$ methyl propanone di HCI suppresses biosynthesis of corticosteroids (*Proc Soc Exp Biol Med* 94/421/57). J Spizizen shows phage infection reduces pyruvate metabolism (*Biochim Biophys Acta* 23/333/57). L Sarda & Co suggest lipase is lipoprotein reacting with Ca ions (*ibid* 264). LC Strong notes relation of biological equilibrium to litter size & spacing & to cancer (*Science* 125/595/Mch 29/57). SA Waksman notes similarity of albomycin & grisein (*ibid* p585, 587). HV Weiss & WH Shipman show biological concentration of cobalt 60 in clams after radiactive fallout (*ibid* p 695/Apr 12).

Clinical: NI Berlin & Co review erythrocyte production, destruction, vol & methods of analysis (*Blood* 12/147/57). PH Bulle suggests reserpine for treating epilepsy (*Proc Soc Exp Biol Med* 94/553/57). SM Garn suggests lower thoracic area for x-ray estimation of body fat (*Science* 125/550/Mch 22/57). AM Master & Co show mean BP of 145/82 (men) & 156/84 (women) in ages 65–106, with no increase with age in healthy persons (*Proc Soc Exp Biol Med* 94/463/57). M Mazen & R Put find insulin useful in methyl Alcohol toxicity (*Acta Physiol Polonica* 9/461/57). MD McCarthy & J Odell find thermal injury suppresses hematopoiesis (*Proc Soc Exp Biol Med* 94/489/57). JC Muhler shows Na fluoride topically reduces dental caries, but not stannous fluoride, or Na N-palmitoyl-sarcosinate (*ibid* p 515). L Revol gives *La therapeutique par la chlorpromazine en pratique psychiatrique* (Masson, Paris, 57, 154pp, 800Fr). LW Roth & J Blair find persisting hypertension after long feeding of pepper, mustard & ginger in combination in various diets (*Proc Soc Exp Biol Med* 94/619/57). EA Swinyard & Co show withdrawal hyperexcitability to meprobamate (*Science* 125/739/Apr 19/57). WP Thompson shows increased emotionality in rats from prenatal maternal anxiety (*ibid* p 698, Apr 12). JE Tracy tells about *The Doctor as a Witness* (Saunders, Phila, 57, 221).

General: CS Blinderman appraises Thomas Henry Huxley 1825–1894 (*Sci Month* 84/171/57). EG Boring considers paradox of free behavior (*ibid* p189). TA Boyd biographizes Charles (Boss) F Kettering in *Professional Amateur* (Dutton NY 57 242pp $4.5). CW Churchman & Co offer an *Introduction to Operations Research* (Wiley NY 57 645pp $12). P Dansereau gives *Biogeography: An Ecological Perspective* (Ronald Press, NY, 57, 369pp $7.5). P deKruif does Dr. J. Ferguson's story in *A Man Against Insanity* (Harcourt Brace, NY, 57, 244pp, $4). LR Dice adds his argument to ecology & overpopulation (*Sci Month* 84/165/57). CC Gillespie has misgivings about pt of view of J Needham's *Science & Civilization in China* (*Amer Sci* 45/169/57). H Grapow offers *Grundriss der Medizin der Alten Agypter III; Kranker, Krankheiten und Arzt* (Academic Verlag, Berlin, 1956, 168pp). M Grotjahn goes well *Beyond Laughter* (McGraw-Hill, NY, 57, 285pp, $6). AC Hardy goes after *The Open Sea: The World of Plankton* (Houghton-Mifflin, Boston, 1957, 335pp, $6.5). JH Hildebrand tells about *Science in the Making* (Columbia Univ Press, NY, 57, 116pp, $3). LS Rodberg & VF Weiskopf describe expts destroying concept of mirror symmetry in atomic structure (*Science*

125/627/Apr 5/57). WK Vivret edits symposium on environmental needs of the aging (*Geriatrics* 12/211/57).

JUNE, 1957

The Big Debate: A Schweitzer's important declaration of conscience on dangers of radiation fall-out appears in full with introduction by N Cousins (*Sat Rev* May 19/57, pp 13–20), with comment by WF Libby, H Brown & J Lear (*ibid* May 25/57, 8–13). M Eisenbud is optimistic on effects of distribution of Sr90 from nuclear blasts (*Sci Month* 84/237/57).

Personality Studies: HP David & HV Bracken note *Perspectives in Personality Theory* (Basic Books, NY, 57, 435pp, $6.5). H Feigl & M Scrivens edit *The Foundations of Science & The Concepts of Psychology & Psychoanalysis* (Univ Minnesota Press, Minneapolis, 57, 346pp, $5). CS Hall & G Lindsey discuss *Theories of Personality* (Wiley, NY, 57, 572pp, $6.5). T Leary makes *Interpersonal Diagnosis of Personality* (Ronald Press, NY, 57, 518pp, $12).

Clinical: AL Barach & HA Bickerman edit *Pulmonary Emphysema* (Wms & Wilkins, Balt., 57, 55pp, $10). JB Chewning edits symposium on cardiovascular disorders in the aged (*Geriatrics* 12/273–338/57). EJ Farris reviews *Human Ovulation & Fertility* (Lippincott, Phila, 57, 159pp, $6.5). M Gleason, R Gossehn & HC Hodge compile *Clinical Toxicology of Commercial Products* (Wms & Wilkins, Balt, 57, 1160pp, $16). BL Gordon edits *Clinical Cardiopulmonary Physiology* (Grune & Stratton, NY, 57, 768pp, $16). R Greene notes wide variation in mental performance in chronic anoxia, with reduction in memory & increase in irritability (*Brit Med J*, 1/1028/May 4/57). M Harrington edits discussion on *Hypotensive Drugs* (Pergamon Press, London, 57, 221pp, $8). HE Himwich edits timely symposium on *Alcoholism* (AAAS, Washington DC, 57, 212pp, $5). H Lisser & RF Escamille prepare *Atlas of Clinical Endrocrinology* (Mosby, St. Louis, 57, 476pp, $19). J Robuck & Co edit *The Leukemias, Etiology, Pathophysiology & Treatment* (Academic Press, NY, 57, 711pp, $13). GD Talbott & Co edit color atlas of *Blood & Bone Marrow Patterns* (Grune & Stratton, NY, 57, 60pp, 145 pict, $12). RHS Thompson & J King note *Biochemical Disorders in Human Disease* (Academic Press, NY, 57, 850pp, $13). A Unugur & Co find 8–16 mgm 1,4-dimethanesulfanyl-oxybutane ("Myleran") consistently & safely effective in chronic granulocytic leukemia (*New Eng J Med* 256/727/Apr 18/57).

Preclinical: H Davis reviews biophysics of inner ear (*Physiol Rev* 37/1/57). CE Dent edits symposium on inborn errors of metabolism (*Amer J Med* 22/671–783/57). A Gottschalk discusses virus enzymes & templates (*Physiol Rev* 37/66/57). RG Grenfell & LJ Mullins edit *Molecular Structure & Functional Activity of Nerve Cells* (Amer Inst Biol Sci, Washington DC, 57, 169pp, $5). AT James & Co fail to find deficiency of essential fatty acids in coronary artery disease (*Lancet* 1/705/Apr 6/57). IM Klotz describes *Some Principles of Energetics in Biochemical Reactions* (Academic Press, NY, 57, 64pp $3). LG Lajtha surveys bone marrow metabolism (*Physiol Rev* 37/50/57). WD McElroy & B Glass give *The Chemical Basis of Heredity* (Johns Hopkins Press, Baltimore, Md, 57, 864pp, $12.5). RC Mellors edits *Analytical Pathology: Treatises in the Perspective of Biology, Chemistry & Physics* (McGraw-Hill, NY, 447pp, $12). JE Mielke & Co find b-aminoproprionitrile

inhibits connective tissue formation (*Proc Soc Exp Biol Med* 94/673/57). WG Overend & AR Peacocke well review molecular basis of heredity (*Endeavor* 16/90/57). TA Rogers & M Kleiber show milk fat synthesis from mammary gland injected acetate (*Proc Soc Exp Biol Med* 94/705/57). RJ Schnitzer & E Grunberg describe *Drug Resistance of Microorganisms* (Academic Press, NY, 57, 395pp, $10). CD Swanson Discusses *Cytology & Cytogenetics* (Prentice Hall, Englewood, NJ, 57, 608pp, $10).

General: G Bergman digs *Philosophy of Science* (Univ Wisconsin Press, Madison, 57, 181pp, $5). NR Campbell brings together science, imagination & art (*Science* 125/803/Apr 26/57). J Gallant discourses on literature, science & shortage of Scientists (*ibid* p787). CL Godske & Co give *Dynamic Meteorology & Weather Forecasting* (Amer Meteorol. Soc, Boston, 57, 816pp, $15). WJ James biographizes Carl Linnaeus 1707–1778 (*Endeavor* 16/107/57). H Jeffreys analyses *Scientific Inference* (Cambridge Univ Press, Cambridge, Eng, 57, 238pp, $5.5). HJ Muller discusses man's place in living nature (*Sci Month* 84/245/57). Unpleasant is the implication of V Packard's *The Hidden Persuaders*, the story of applications of MR, motivational research (McKay, Phila, 57, 211pp, $4). A Rapoport adds push to scientific approach to ethics (*Science* 125/796/Apr 26/57). Startling is CA Scarlott in analysing changing energy picture (*Sci Month* 84/221/57). PB Sears comments on Cultural aspects of floods (*Science* 125/806/Apr 26/57).

A METHOD TO RECORD PROTHROMBIN TIME

JACK G. BISHOP, J. LESTER MATTHEWS, AND
ALFRED W. RICHARDSON

Since the work of Howell (1914) many investigators have been interested in the determination of prothrombin time. Numerous methods have been devised to detect the precise time of clot formation. Subjective determinations of viscosity changes such as the tilting method (Quick, 1945), nichrome wire method (Tocantins, 1955), glass rod method (Kato, 1940), and syringe drop method (Market and Winterstein, 1953) have been used. Photoelectric determinations of the end point have been used, (Nygaard, 1941; Rheese, *et al.*, 1955; Lasner, *et al.*, 1950). Results of different methods are not comparable due to variations in rate and degree of agitation, temperature variations, and subjective errors in determining the end point.

With these factors in mind, we have developed a method to record prothrombin time that has the advantages of (1) uniform rate of agitation; (2) temperature control during clotting; (3) minimum of subjective error in determining the end point; and (4) permanent records.

Methods

The instrument is a modification of that used in the blood coagulation method of Richardson and Bishop (1953). The apparatus used, figure I, is composed of a 110 V a.c. motor attached to a gear that oscillates a shaft back and forth through 90°. A platform is attached to the shaft in a position which will swing it 45° up and down from horizontal. A full oscillation occurs every one second. A constant temperature plasma container and a mercury switch are mounted on the platform. The switch is mounted in reverse position to the platinum electrodes, figure II, so that the circuit is completed only when the electrodes are 45° above the horizontal. Thus, conductivity of the clotted plasma and not the liquid portion is recorded.

Plasma containers, constructed from polystyrene plastic tubes, 20 mm. × 10 mm., were inserted into a polyethylene friction cap of the constant temperature chamber. Heating chambers, similar to those of Rhees, Ellerbrook, and Brown (1955), were constructed from polystyrene plastic tubes. This permits visual observation of the clotting process. A temperature of 37° C. was maintained during the clotting process.

Conductive electrodes were constructed by cementing two platinum pins

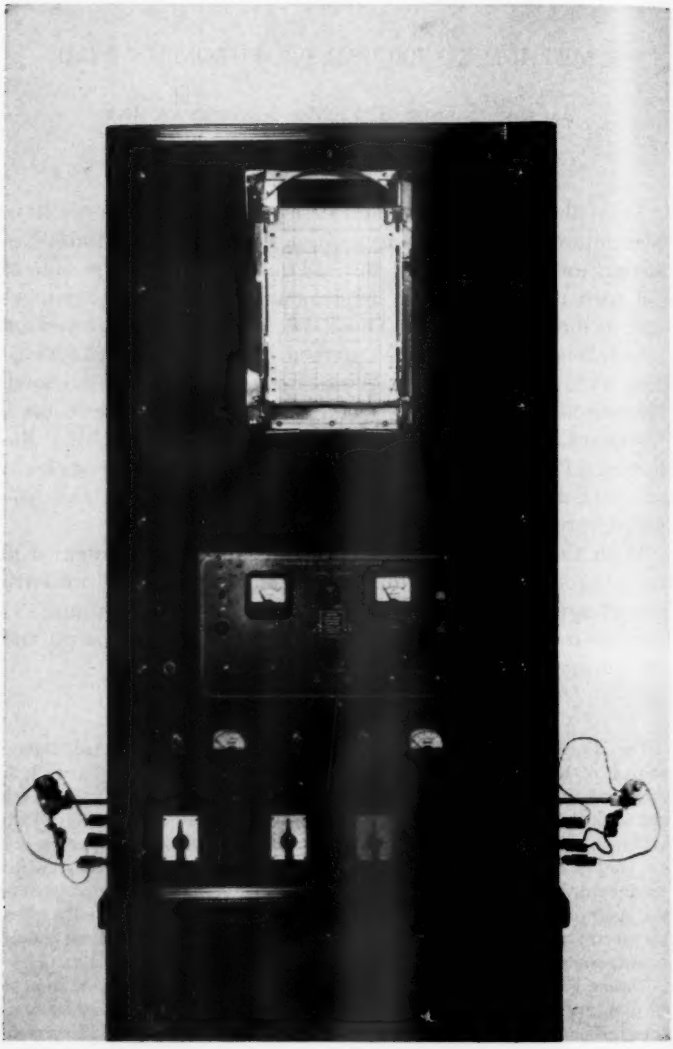

FIG. I. Photograph of complete prothrombin meter. Esterline-Angus recorder and battery eliminator are mounted above oscillating motor. Rotating platform, heating chamber, and mercury switch are shown on side.

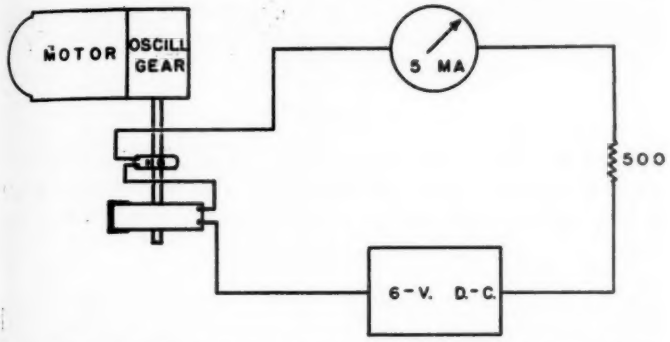

FIG. II. Schematic diagram showing oscillating motor, Esterline-Angus, D. C. Source, electrodes and mercury switch. A 500 ohm resistor is used to protect the movement of the recorder.

through a plastic coated stopper so that the heads touched the inner surface 3.5 mm. apart and protrude 1 mm. through the stopper. The heads of the pins present a smooth surface to the rotating plasma and facilitate cleaning. Cleaning of electrodes is effected following each determination, using a detergent saturated gauze followed by successive distilled water rinsing. A new thoroughly clean and dry plasma cell is used for each determination. Cells are subsequently cleaned, as above, for reuse.

Prothrombin times were recorded using a modified Quick one-stage method (1945). 0.1 ml. test plasma, 37° C., was placed in the plastic chamber and allowed to equilibrate for five minutes. 0.2 ml. thromboplastin-calcium mixture, Simplastin, at 37° C. was delivered by means of a syringe pipette to the chamber. The electrodes were positioned and the Esterline-Angus Recorder and stage rotation were started immediately by means of a foot switch.

The effectiveness of this method in determining a critical end point was evaluated by 30 analyses of diagnostic plasma (Warner-Chilcott). In addition, prothrombin time of fresh human plasma samples, furnished by Wadley Blood Bank, was measured. Thirty determinations on samples from four patients were used. The Hematology Department of Baylor University Hospital furnished plasma from 26 patients for comparative studies. Duplicate prothrombin time determinations were made, using the recording method, and these data were compared with the results obtained by the Hematology Department, which used the visual method.

Prothrombin time on Hyland Laboratories prothrombin Reference Standard diluted to 20, 40, 60, 80, and 100 per cent of standard was determined to ascertain the reliability of the method with different concentrations of prothrombin. A modification of the Owren method (1949) was used in this series. 0.1 ml. of prothrombin-free beef plasma (37° C.) was added to 0.1 ml. of standard plasma (37° C.) and was allowed to equilibrate for five minutes in

the plastic chamber. This assured a constant temperature and complete mixing of the samples. 0.1 ml. of thromboplastin-calcium mixture (Simplastin, at 37° C.) was delivered and recording was begun immediately.

Results

Figure III shows two typical records obtained using equal aliquots of human plasma and Simplastin. The first abrupt change in conductivity was used to denote the end point. Prothombin time was 10 seconds for each sample.

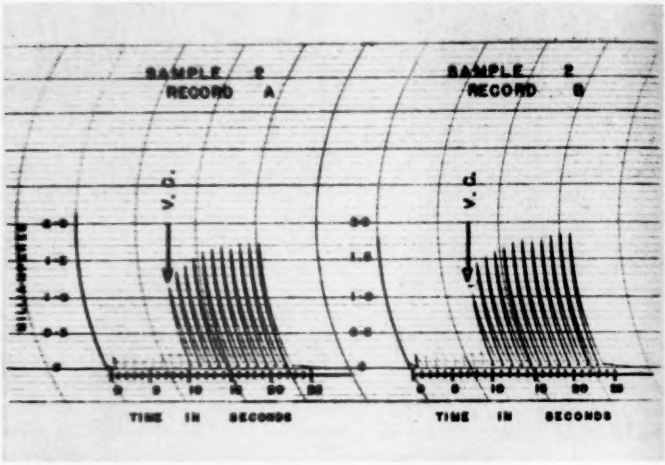

FIG. III. Two records obtained from equal volumes of the same patient's plasma plus Simplastin. Time in seconds on the abscissa and conductivity on the ordinate. V.C. represents clot formation.

Figure IV demonstrates prolonged prothrombin time on plasma obtained from a patient whose condition had been previously diagnosed as hypoprothrombinemia. Clotting time for each prothrombin sample, records A and B, was 40 seconds.

In order to determine the reliability of this method determinations were made on 30 standardized plasma and 30 human plasma samples. These data are shown in table 1. The prothrombin time for the standardized plasma ranged from 11 to 15 seconds. The mean prothrombin time was 12.8 seconds. The standard error of the means was ± 0.17 seconds. The mean for the human

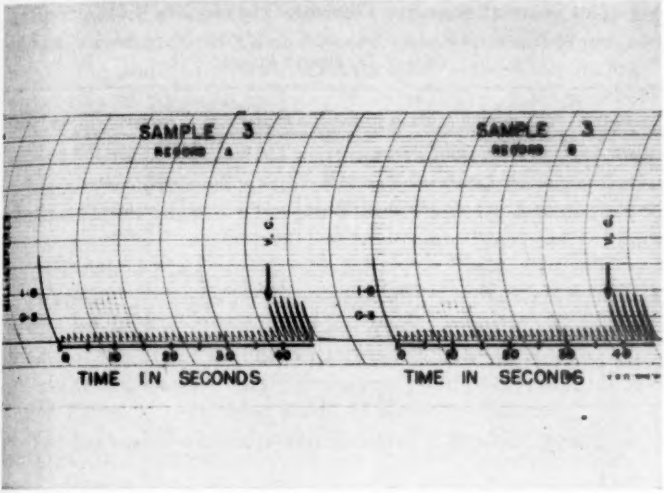

FIG. IV. Dual records from a patient with hypoprothrombinemia. V.C. represents clot formation.

plasma was 14.8. The standard error of the mean was ± 0.18 seconds.

Table 2 demonstrates the comparison of the average prothrombin time obtained by experienced technicians using the visual method and the recording technique. Each sample represents plasma obtained from different hospital patients in the course of routine serological determinations. The recording and visual determinations were made simultaneously on aliquots of the same patient's plasma. It is of interest that prothrombin time is generally shorter with the recording method.

The results obtained by the recording method for the modified Owren prothrombin test are shown in figure V. Dilutions were made and allowed to equilibrate to 37° C. before the thromboplastin-calcium mixture was added. Each point on the figure represents an average of 8 samples.

Discussion

The records obtained by this method were comparable to an inverted photoelectric record as described by Nygaard (1941). The point at which there was an abrupt change in the recorded

TABLE 1

Comparative Studies of Fresh Plasma Obtained From Four Donors and Five Lots of Diagnostic Plasma

Fresh Human Plasma			Diagnostic Plasma (Warner-Chilcott)		
Patient	Sample	Time in Sec.	Plasma	Sample	Time in Sec.
Donor A	1	14	Lot 1	1	15
	2	14		2	13
	3	14		3	14
	4	14		4	14
	5	14		5	14
	6	13		6	13
	7	16			
	8	16	Lot 2	1	11
	9	15		2	14
	10	14		3	13
				4	13
Donor B	1	16		5	13
	2	16		6	12
	3	16			
	4	16	Lot 3	1	12
	5	15		2	12
				3	13
Donor C	1	14		4	12
	2	14		5	12
	3	16		6	13
	4	14			
	5	15	Lot 4	1	13
				2	14
Donor D	1	17		3	13
	2	15		4	12
	3	14		5	12
	4	15		6	12
	5	14			
	6	15	Lot 5	1	14
	7	15		2	12
	8	15		3	12
	9	14		4	12
	10	15		5	13
				6	12
Mean = 14.8 ± 0.18			Mean = 12.8 ± 0.17		

ordinate was used as the end point. The point corresponded to the point selected for the photoelectric method. It was designated as visual clot (V.C.) on figures III and IV. The magnitude of change was sufficient to provide an easily discernible end point of the first fibrin formation. Additional points may be obtained which represent the end of fibrin formation. No physiological significance has been attached to the difference in conductivity of different plasma.

A maximum of one second variation may be attributed to the speed of rotation of the plasma chamber. Experimental variation

using standardized plasma showed a variation from 11 to 15 seconds. The mean was 12.8 ± 0.17 seconds. Standardized diagnostic plasma and Simplastin, assayed by Warner-Chilcott, has a mean plasma prothrombin time of 13 ± 1 second. From laboratory to laboratory, plasma prothrombin time may vary from 12 seconds (Quick, 1951) to 17 seconds (Maple and Wright, 1950) using the standardized plasma. Repeated recordings of prothrombin time from 4 healthy human patients gave a range of 13 to 17 seconds with a mean of 14.8 ± 0.18 seconds. Maximal variation in each donor group was 3 seconds.

In comparative studies of the recording method and the visual tilting method prothrombin times were generally shorter with the recording method. This variation would be anticipated since the recording method has a sharp end point at the beginning of fibrin formation. Samples 8 and 17 were exceptions.

The log log relationship seen in figure V demonstrates that the

TABLE 2

Comparison of the Average Prothrombin Time Obtained by the Recording Method and Workers Experienced With the Visual Tilting Method

Patient	Time in Sec. Rec. A.	Rec. B.	Time in Sec. Visual
1	22	22	35
2	10	10	15
3	40	40	46
4	11	11	16
5	21	22	33
6	29	31	42
7	12	12	20
8	35	32	30
9	31	31	44
10	16	15	22
11	21	21	32
12	16	16	26
13	15	15	23
14	30	30	45
15	19	19	29
16	8	9	15
17	37	37	30
18	19	20	28
19	16	15	20
20	9	10	17
21	9	9	15
22	13	13	20
23	8	9	16
24	25	25	29
25	16	16	23
26	13	13	23

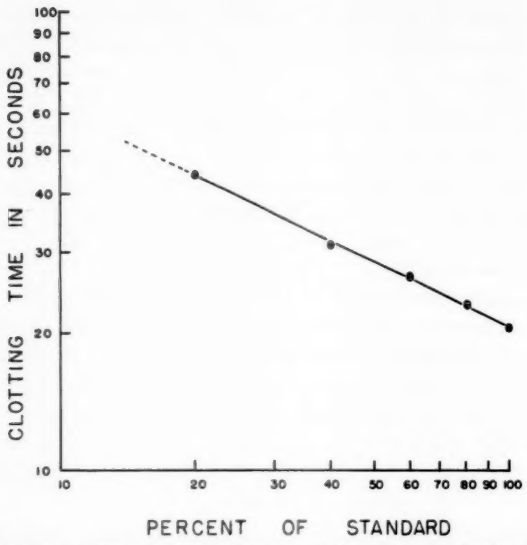

FIG. V. The log log relationship between dilutions of standardized plasma and Simplastin. Each point represents an average of eight recordings. Maximal variation for each point was 3 seconds.

end points selected are related to prothrombin concentration. Prothrombin concentrations as low as 20 per cent were sufficient to produce an adequate clot for conductivity measurements.

Preliminary results indicate that this method can also be used for proaccelerin determinations.

Summary

A new method to record prothrombin time based upon conductivity changes during clot formation has been described.

The recording method has the following advantages: (1) sharp end point, therefore a minimum of subjective error; (2) constant rate and degree of agitation; (3) reproducible permanent recordings; (4) considerable accuracy, as shown by the relative low standard of error of the mean using repeated sampling of the same plasma; and (5) controlled temperature during the clotting process.

Records comparable to those of the photoelectric method were demonstrated.

Prothrombin concentrations as low as 20 per cent were sufficient to produce an adequate clot for conductivity measurements.

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[b] Department of Physiology, Baylor University College of Dentistry, Dallas.

[c] Department of Physiology, Baylor University College of Dentistry, Dallas.

[d] Department of Physiology, St. Louis University School of Medicine.

[e] Simplastin and diagnostic plasma were furnished by Warner-Chilcott, New York, New York.

[f] Reagents for the modified Owren prothrombin test were furnished by Hyland Laboratories, Los Angeles, California.

THE STATUS OF VENEREAL DISEASE CONTROL IN TEXAS

ALLAN R. DOANE[a]

The status of venereal disease control in Texas, as elsewhere in the nation and throughout the civilized world, has been in a state of flux for the past fifteen years; nor has the situation resolved itself. The wave of optimism which followed the advent of the sulfonamides and penicillin, their effectiveness in the treatment of gonorrhea; and penicillin in its effectiveness in the treatment of syphilis, was too quickly replaced by gross complacency. Physicians including public health doctors eagerly proclaimed that syphilis and gonorrhea were solved problems! Budgets were slashed, personnel reduced, control programs curtailed. Today we are returning more actively to programs of control, and are reaping the harvest of this misplaced optimism.

We recognize unique factors in venereal disease control which are not wholly Texan in nature, and which have catered to inordinately high venereal disease rates. We have, for example, a southern international boundary of over nine-hundred miles; and within the state, there are fifty-three military installations with thousands of young men, at the most libidinous period of their lives, recreating throughout and beyond the state. The most disconcerting development, and one which greatly complicates control activity, is the shift from older to younger age groups involved with the venereal diseases. Of the 266,000 persons in the nation diagnosed with early syphilis and gonorrhea last year, fifty-five per cent were teen-agers and young adults. Studies made in 1953 and 1955 indicate alarming increases in venereal disease incidence after the age fourteen; with more females being infected at eighteen than at any other age.

Today, venereal disease morbidity statistics are such an incomplete entity that we have no accurate statistical evaluation of the extent of the problem. In 1943, we commenced using penicillin. It is a matter of statistical record that 575,000 persons in Continental United States were reported with syphilis; whereas, in 1955, 122,000 persons were reported. This represents

a drop in rate, per 100,000 population, from 447 to 76. Reassuring as this decline may be, the statistical evidence is still sufficient to indicate that venereal disease is a major public health problem in Texas as in the nation.

In 1943, the vast majority of patients suffering from syphilis and gonorrhea, as well as the other venereal infections, were diagnosed and treated at the public clinics. Even at that time, no self-respecting private physician wished to become known as a "clap doctor," or to have patients with syphilis associating in his office with his "respectable patients." As result of public education, the taboo on venereal disease as something "unmentionable" has greatly lessened. Because of the simplicity and effectiveness of penicillin treatment, the venereal disease patient is now welcomed by the private practitioner whom we estimate treats more than one-half of the patients formerly treated, and epidemiologically investigated, at the public clinic. There has been an understandable reluctance on the part of the private physician to make his private patients accessible for epidemiologic investigation. We find in Texas that, by and large, we do not have this access; nor have we sufficiently encouraged the physician to give complete morbidity reports on these diseases. Nevertheless, and quite rightly, the morbidity reporting of venereal diseases is, in Texas, a legal requirement.

From the point of view of economy, and with the transition that is rapidly occurring, one can foresee the time—except in our largest metropolitan areas—where the diagnosis and treatment of the venereally infected will be accomplished completely by the private physician. In our larger cities, the trend will and should be to relocate our public clinics in the Out-Patient Clinics of public, preferably teaching, hospitals. It is apparent, then, that our most pressing problem in control is now, and in future will be even more, the adjustment of our state and local public health departments to give consultant services to the private physicians; and, in turn, through state or local medical societies, to formulate an effective plan so that the state will receive complete morbidity statistics. Thus the local health department, through the public health physician, the public health nurse, or the specially trained male Interviewer-Investigator, may achieve the necessary epidemiology in locating venereal disease contacts.

Case-finding and contact investigation is the only known

means at our disposal for uncovering reservoirs of infection and eliminating them, and we shall not be successful in controlling—much less eliminating—our greatest hazard "syphilis" without the active cooperation of all concerned in implementing these public health aspects of control. We suggest that public health physicians and our colleagues in therapeutics will have to get together, especially through local medical societies, and develop, by constructive discussion, a workable plan of cooperation.

From recent reports, we have been learning that the incidence of infectious syphilis is rising. In some areas of Texas, we have found this is true, but in some other areas, we have noticed a marked decline not only in infectious syphilis, but in all venereal disease morbidity. Accepting the inevitable occasional sporadic rise, which characterizes the epidemiology of all communicable diseases, we contend that there exists much infectious syphilis which a carefully directed program could reveal. It is remarkable, without exception, that wherever in the state we have intensified case-finding techniques, the morbidity of infectious syphilis has risen. In those areas where a decline has been noted, we find that very little is being done in active case-finding. In both situations, and especially in the past year, there has been a remarkable decline in morbidity reporting from private physicians. From our experience, we suggest that the incidence of infectious syphilis in many areas could be used as the criterion of an effective program. To illustrate with brief equations:

Effective case-finding = increased incidence of infectious syphilis

Ineffective or no case-finding = decline of infectious syphilis.

A specific example of intensifying case-finding techniques was applied last year in Houston; and as a direct result, has uncovered some of the most interesting and sizeable epidemiologic patterns appearing in the nation today. This project has revealed that there is much infectious syphilis in Houston—as there probably is elsewhere—but before the endeavors were made, we heard there was no infectious syphilis in that area. The study revealed a remarkable pyramid involving over three-hundred contacts in a chain from an original case of early latent syphilis. Of these contacts, sixty-four were found to have syphilis; and of these, fifty-nine were infectious. This particular chain has not

yet been completely resolved, but it does emphasize the direct result of concentrated teamwork in action.

To exemplify once more how unreliable venereal disease statistics are in assessing the actual problem, we found in one county in Texas, during 1956, five cases of syphilis were reported to the local health officer from private physicians. Upon checking the state laboratory in that area, a specially conducted survey revealed that 9,468 blood serologies were performed for private physicians; and of these, 503 were definitely reactive, and 351 were doubtful. The total would be considerably higher if we had access to private laboratories in that area. Surely, this discrepancy is too great to be covered by the "false positive" category!

TABLE 1

Venereal Diseases in Texas	1954	1955	1956
Infectious syphilis	1,040	775	1,589
Other syphilis	3,491	2,516	2,860
Gonorrhea	20,692	19,171	15,062
Other Venereal Diseases	228	262	157
TOTAL	25,451	22,724	19,668

The remarkable features of this table are the increase in infectious syphilis and the decrease in gonorrhea last year over the two preceding years, and the slight annual decrease in total morbidities in the three years reported. We are quite certain that the decrease in morbidity reporting of gonorrhea is the result of the decline in reporting of these on the part of the private physicians which also expresses itself as a decline in the total morbidities. We reiterate, however, that a major factor in the increase of infectious syphilis has been the result of intensified case-finding. Chancroid, lymphogranuloma venereum and granuloma inguinale account for the "Other Venereal Diseases" reported; but in contrast they are so insignificant compared to gonorrhea and syphilis that they do not merit separate consideration in a general report on present-day venereal disease infections.

As the transition from the clinic for diagnosis and treatment to the private physician's office becomes more complete, an increasing number of patients will be evaluated by those who are not specialists in the field of syphilology and venereology. In a

well-organized clinic, in the past, the Diagnostic and Therapeutic Director was usually a skilled consultant in his field who had recourse to consultants of practically all the specialities of medicine. Since the medical graduate today is almost certain to encounter, in practice, problems in syphilology, as well as the other venereal diseases, he should be familiar with adequate criteria of diagnosis, treatment and cure. This is also a strong indication why clinical facilities for the venereal diseases should be housed in the Out-Patient Clinic of a general teaching hospital where, at least, the student and intern may have access to available clinical material. He should be prepared in medical school to accept, fully, his public health responsibilities in dealing with these communicable diseases. There should be available refresher courses and courses in up-to-date orientation for practicing physicians who need and very often want this assistance. The services of the State Division of Venereal Disease Control should be better prepared to give comprehensive consultant service for general practitioners throughout Texas who are already encouraged to send their problems in diagnosing and treating the venereal diseases, especially syphilis, to this Division. The physician who will receive and treat, in complete confidence, a patient with infectious syphilis regarding the relationship as involving only the patient and himself might be compared to the surgeon who simply removes a tumor neglecting, if it is malignant to "perform a radical." In the former situation, he may be even more remiss because he neglects others whose welfare may, indeed, be more precious to the patient than his own.

Gonorrhea, although greatly relieved of its complications, is today not much less common than in the past. However, infectious syphilis has declined greatly; but where active and efficient case-finding techniques are pursued, it still is revealed in numbers sufficient to cause concern and to merit effective action.

In regard to syphilis, it is very difficult to understand the widespread complacency not only in regard to diagnosis and treatment, but most especially toward epidemiology directed at uncovering reservoirs of infection. It is remarkable that this complacency should be adopted by so many physicians. Wherever eradication of disease has been achieved, it has been traditional for the medical profession to view, with alarm, the occurrence of a single case, and for the attending physician and public health

authorities to collaborate and act promptly in curtailing spread. One case of small-pox anywhere in the nation today, would be a matter for precipitate and coordinated action on the part of the physician, local, state and federal health as well as other authorities; and with notable repercussions internationally throughout the civilized world. In the United States, we claim typhoid fever and diphtheria are no longer epidemic diseases, but their sporadic occurrence today calls for prompt, suppressive, preventive measures on the part of all concerned. No disease in the history of civilized man has matched, in magnitude, the scourge of syphilis. It is an acute infectious disease at onset, with a relapsing tendency, characterized by stages of complete asymptomatic latency, which if untreated very often becomes a widespread constitutional disorder of great chronicity. There is no symptom it cannot cause; no tissue of the body which it may not invade; and no other disease is related so fundamentally to the human emotions and instincts. It may resemble any of the skin conditions known to the dermatologists. It may invade the skeleton with deforming and crippling effects; the central nervous system causing meningitis, blindness, deafness, insanity, and paralysis. It is the underlying cause of much cardiovascular disease, and is responsible for that most tragic of all disease entities—congenital syphilis. In its wake, we have the maimed, the halt, the blind, the deaf, the insane, and innocent children marked at birth. Add to this the anguish, the shame, the divorce, the disrupted homes, and even suicide which syphilis has motivated, and we can appreciate the scope of this monstrous disease. If the professional index of suspicion for syphilis has been much too low in the past, it is seriously so today. As in the time of Sir William Osler, and as he so accurately directed, "syphilis enters into every differential diagnosis." He admonished his medical students "to know syphilis and all else in medicine would be added." No "wonder drug," no control program has yet replaced these sound propositions.

We have not yet been successful in controlling the morbidity of gonorrhea. Many medical authorities contend that we will not control either syphilis or gonorrhea by treatment alone. Because of its short incubation period and the predisposition of the host to reinfection, it is obviously more difficult to control the morbidity of gonorrhea than syphilis. With sulfonamide therapy, we

noticed a great reduction in the complications of gonorrhea, but there is considerable evidence to show that these may very well have been partly the result of former treatment procedures. In the late thirties, sulfonamide therapy was the ultimate treatment for gonorrhea, but its optimistic future was, in the words of Dr. Warfield Garson, Director of the Venereal Disease Experimental Laboratory, Chapel Hill, North Carolina, "blasted by the biochemical capabilities of the gonococcus to become resistant to these drugs." In the early mid-forties, our optimism and enthusiasm was greatly renewed with the effectiveness of penicillin in treating gonorrhea; and with this agent, complications all but disappeared from our hospital wards and clinic practice. Nevertheless, gonorrhea morbidity, among the communicable diseases, is still one of the greatest. Dr. Garson, in a recent paper, has significantly suggested that the dosage of penicillin adequate to treat effectively anterior urethritis in the male, in almost one-hundred per cent of the cases, may very well be insufficient to treat the gonorrhea in the female. His findings support this hypothesis. In his paper, Dr. Garson indicates many factors inherent in the female which make diagnosis and adequate treatment more difficult than in the male, and states that pending clarification, new knowledge and techniques, we should raise our dosage for the routine treatment of gonorrhea at least in females. In their studies, Preston and Dunsworth of the Richland County Health Department, Columbia, South Carolina, have concluded that 1,800,000 units of PAM penicillin is necessary for an acceptable rate of cure of gonorrhea in females. Dr. Carl Hookings of the Shelby County Health Department, Memphis, Tennessee, has an impressive report concerning his studies with a mixture of PAM and bicillin totalling 1,800,000 units dosage which is applicable not only to the treatment of early diagnosed gonorrhea in the female, but also to the prophylactic use of penicillin of all other females brought under observation. Apparently, current gonorrhea therapy is inadequate for satisfactory control purposes.

We have approached the control of other communicable diseases on the basis of a knowledge of their known modes of transmission. It is direct and impersonal to chlorinate water, to pasteurize milk, to eliminate mosquitoes, and to stimulate antibody formation with specific vaccines; but in the venereal diseases,

whose mode of transmission is chiefly sexual, and in the absence of a satisfactory vaccine, control becomes deeply personal and, therefore, very intricate and involved. Those public health personnel engaged upon this most difficult and challenging aspect of all epidemiology require a sufficient background in education, specially developed skills, proven aptitude, initiative, tact, integrity; and in a sense, must be dedicated to their work. The private physician, even if he is adept and frequently he is, is too busy for this time-consuming work. The public health physician, since this is his prerogative and specialty, must provide the private physician with assistance in which he has confidence and with which he will lend cooperation in bringing his patients to contact investigation. To many, this is a new idea, and to many a distasteful innovation; but because it is logical and the best we have to offer, it must somehow be developed!

My experience with physicians in Texas has been so refreshing and stimulating that I feel very strongly that we, in public health, have only to meet and discuss with private physicians our mutual problems; and together, in the light of modern knowledge, solve them. I can see this as the only means of implementing a program of venereal disease control adequate for our great state. Let us, in Texas, recognize the changes which have taken place; and let us, in the face of this unique challenge, lead our nation in venereal disease control.

[a] Medical Director, Division of Venereal Disease Control, Texas State Department of Health, Austin.

THE SIGNIFICANCE OF ANTIBACTERIAL IMMUNITY IN DIPHTHERIA

LUDWIK FLECK[a] AND ANNA KUNICKA

Epidemiological observations prove that diphtherial infection may occur in spite of vaccination with anatoxin and even in spite of a high level of antitoxin in blood. This problem has been considered in many papers. Raffel reports 63 per cent vaccinated children among the diseased ones during the epidemic in Baltimore (1944). Rendu noted similar percentages in Switzerland and France in the years 1943–1945. Hartley, Anderson, Grant and others reported (1950) 30–50 per cent vaccinated persons among the diseased in Tyneside and Dundee. Moreover, out of 264 cases in Tyneside 32 (= 12 per cent) had an antitoxin level above 0.1 U/ml., half of them had even more than 1 U/ml. at the beginning of the illness. Prohazka (1937) described diphtheria in 21 vaccinated children with antitoxin levels above 0.04 U/ml., six of them with 0.2 U/ml. at the beginning of the illness. In the material from Warsaw during 1955 and first half of 1956, Dr. Malyszko found about 30 per cent vaccinated out of 569 patients.

On the other hand, there exist observations that infection can miss nonvaccinated persons, with no antitoxin in blood though being actually carriers of virulent bacilli or living in proximity to diphtheria patients.

Thus, no absolute relation seems to exist between the antitoxin level in blood and the susceptibility to diphtheria infection. The vaccination with anatoxin only, unsatisfactorily prevents the acquiring of diphtheria though it surely appeases the course of an acquired disease.

Therefore, beside the antitoxic, an antibacterial immunity has been taken into consideration. We quote Phair (1942), Huang (1942), Ørskov (1944), Orr-Ewing (1946) as the earlier authors on this problem. The subject has been summarized by Raffel (1949).

In the current decade about ten authors wrote on the significance of antibacterial immunity in diphtheria. Frobisher and

Parsons (1950), based on their animal experiments, underlined "the desirability of including endotoxins in toxoid mixtures used in the immunization of human beings." Unfortunately, these authors have not taken into consideration the serotypes of C. diphtheriae, although it would be expected that the antigenic differences between the serotypes were important for the specific immunity.

In 1954 Bowen, Wyman, McComb found that "following immunization with washed cellular antigens, guinea pigs are shown to be partially protected against challenge by virulent C. diphtheriae, but not against toxin." But they express the opinion that an addition of cellular antigens to the vaccine may make it toxic or allergy-inducing. Lautrop (1955), basing himself on his own experiments, came to the conclusion that "immunization of guinea pigs with live cultures of an atoxic strain of C. diphtheriae protected about 50 per cent of the animals against a deadly infection with a culture of a toxic strain of the same serotype." He found also a certain inter-typical antibacterial immunity and claims that "probably antibacterial immunity is not type-specific" because of common secondary antigens of the K-kind. Maitland, Marshal, Petrie and Robinson (1952) investigated the effect of an anti-gravis serum on experimental infection and in the treatment of patients. They "suggest, that the role of the antibacterial factor in the pathogenesis and control of diphtheria merits investigations."

Thus, the problem of using bacterial substances for vaccination has been several times dealt with, but not solved until now. Further experimental contributions may be of value.

Materials and Methods

1) Four series of experiments on animals were performed. The first series contained 36 guinea pigs, divided into 4 groups, each involving 9 animals. The first group was immunized with C. diphtheriae strain Nr. 24, the second with strain Nr. 25, the third with strain Park-Williams Nr. 8, the fourth remained non-immunized as a control.

The immunization consisted in injections of bacterial suspension (1 billion in 1 ml.) from 48-hour cultures on Löffler medium, killed with formalin and twice washed with saline in order to remove toxin. The animals received 6 subcutaneous injections at 3 day intervals, amounting from 0.5 ml. to 1.5 ml. of the suspension. The 7th injection (3 ml.) was given intraperitoneally 7 weeks after the 6th injection.

The three strains represented 3 different serotypes, all of them toxigenic.

Nr. 24 belongs to group gravis, Nr. 25 and Park-Williams Nr. 8 are mitis. Ten days after the last injection, the animals were bled (2–3 ml.) in order to determine the level of antitoxin and agglutinins. Antitoxin has not been found, not even in 1/400 U/ml. (Jensen); agglutinins for the homologous strain reached the level 1:50–1:180.

Thirteen days after the last injection, twelve animals (three in each group) received antitoxic horse serum, 25 AU/250 g. each. Twelve other animals (three in each group) were given 50 AU/250 g. each. On the next day followed the challenge by subcutaneous injection of 0.5 ml. suspension (3 billion in 1 ml.) of living bacilli, washed twice with saline. Three animals in each group received the homologous strain, six other the heterologous strains. All killed animals were thoroughly examined for specific changes. The design of the experiment is shown in table 1.

The second series of experiments, of the same design, contained 108 guinea pigs. The challenge dose was given 24 days after the last inoculation. The average agglutination titer of the bacteria inoculated animals was 1:267.

The third series was done with 8 guinea pigs inoculated with living atoxic bacilli of the strain C. diphtheriae 1174 (Freeman) and 6 control animals. The challenge of all 14 was performed with the same strain 1174 made toxic by the action of the Freeman phage B. The average agglutination titer of the 8 inoculated animals for C. diphtheriae 1174 was 1:315.

The fourth series contained 16 guinea pigs inoculated with the living atoxic Freeman strain Nr. 1174, and 16 control animals. The infection consisted in subcutaneous injection of the toxic strain Nr. 25, of the same serotype as the used Freeman strain, from a 24 hour culture, twice washed to remove toxin.

2) Some experiments on volunteers (20 persons) were performed, to establish the intensity of response to injection of killed and washed C. diphtheriae. Three to twenty million killed bacilli given subcutaneously provoked no visible reaction, or a very weak reaction. The immunological response, measured by means of the agglutination titer, was very small. Larger quantities of bacilli had to be injected intramuscularly. About 500–700 million were found to be a proper dosis: the local reaction was not too large, the agglutination titer rose to about 1:100. The general reaction consisted in an insignificant rise in temperature and transitory weakness. No hypersensitivity to diphtheria antigens could be found during six-month observations: the inoculated persons tolerated subsequent subcutaneous injections as well as non-inoculated ones. The "booster effect" was rather small.

A combined toxoid-bacilli vaccine was prepared. It consisted of a mixture of adequate doses, according to age, of alum precipitated toxoid (anatoxin), 60 Antig.U. in 1 ml. and bacilli suspension. The suspension was made from 48 h. culture on Löffler medium, killed with 2 per cent formalin (48 h.) and twice washed with saline solution. It was adjusted to 700 mil. bacilli in 1 ml., checked for sterility and toxicity; 0.5 per cent phenol was added afterwards. We used the toxic strain C. diphtheriae Nr. 24 as a representative of the most common serotype. We made 3 intramuscular injections at 2-week intervals (anatoxin + 0.5 ml. suspension, anatoxin + 1 ml. suspension, anatoxin + 1.5

TABLE 1

	Group 24 9 Guinea Pigs Immunized with Strain 24 (Gravis) Jan. 17–Mar. 22 The Average Agglutinin Titer 1:111.8			Group 25 9 Guinea Pigs Immunized with Strain 25 (Mitis) Jan. 17–Mar. 22 The Average Agglutinin Titer 1:178.6			Group PW 8 9 Guinea Pigs Immunized with Strain PW 8 (Mitis) Feb. 3–Mar. 22 The Average Agglutinin Titer 1:48			Control Animals 9 Guinea Pigs		
	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs	3 Guinea Pigs
Without antitoxic serum	Nr. 1	Nr. 4 died: 9/10 Apr.	Nr. 7 died: 6 Apr.	Nr. 10	Nr. 13 died: 8 Apr.	Nr. 16 died: 6 Apr.	Nr. 19 died: 6 Apr.	Nr. 22 died: 8/9 Apr.	Nr. 25 died: 8/9 Apr.	Nr. 28 died: 11 Apr.	Nr. 31 died: 9/10 Apr.	Nr. 34 died: 6 Apr.
25 AU/250 g. antitoxic serum given 4 Apr.	Nr. 2	Nr. 5	Nr. 8 died: 6 Apr.	Nr. 11	Nr. 14	Nr. 17	Nr. 20	Nr. 23 died: 12 Apr.	Nr. 26	Nr. 29	Nr. 32 died: 7 Apr.	Nr. 35
50 AU/250 g. antitoxic serum given 4 Apr.	Nr. 3	Nr. 6	Nr. 9	Nr.12	Nr. 15	Nr. 18	Nr. 21	Nr. 24	Nr. 25	Nr. 30	Nr. 33	Nr. 36 died: 6 Apr.
Infected 5 Apr. with strain	24	25	PW 8	25	24	PW 8	PW 8	24	25	24	25	PW 8

Blood examination before antitoxic serum administration: no antitoxin.
Challenge dose: 1.5 billion living bacteria.

ml. suspension), blood was examined before the first and 1 month after the last inoculation.

The combined vaccine ("Anabac" = Anatoxin + bacilli) was used in 39 children and 2 adults.

Results

1. *Experiments on guinea pigs.*

The results of the first series of animal experiments (table 1) can be summarized as follows:

Kind of Immunization	Number of Animals	Died
None	3	3
Antitoxic (25–50 AU/250 g.)	6	2
Antibacterial		
a) homotypic	3	1
b) heterotypic	6	6
c) total	9	7
Combined (antitoxic + antibacterial)		
a) homotypic	6	0
b) heterotypic	12	2
c) total	18	2

Thus, in the conditions of this experiment, we received the best results with the combined (antitoxic + antibacterial) homotypic immunization. The second in effectiveness were given by the combined (antitoxic + antibacterial) heterotype immunization, then follow: the only antitoxic immunity, and the only homotypic antibacterial immunity. The heterotypic immunization alone appeared to be worthless.

The second series of animal experiments, of the same design as the first, but with 108 guinea pigs, gave the following results:

Kind of Immunization	Number of Animals	Died
None	9	9
Antitoxic (25–50 AU/250 g.)	18	5
Antibacterial		
a) homotypic	9	9
b) heterotypic	18	17
c) total	27	26
Combined (antitoxic + antibacterial)		
a) homotypic	18	0
b) heterotypic	36	0
c) total	54	0

Thus, we got also in this series the best results with the combined (antitoxic + antibacterial) immunization regardless

whether homotypic or heterotypic. Then follows the only antitoxic immunity. The antibacterial immunization alone appeared in this experiments worthless.

In the third series of experiments (inoculation with living atoxic strain Freeman 1174, and infection with the same strain made toxigenic by phage action, according to Freeman and Morse) 3 control animals (out of 6) and only 1 inoculated (out of 8) died.

The fourth series (inoculation with living atoxic strain Freeman 1174, infection with toxic strain Nr. 25 of the same serotype) revealed: 9 control animals (out of 16) and only 3 inoculated (out of 16) died.

A joint summary of the very similar first and second series results shows:

Kind of Immunization	Number of Animals	Died
None	12	12 = 100 %
Antitoxic (25–50 AU/250 g.)	24	7 = 29.1%
Antibacterial	36	33 = 91.6%
Combined (antitoxic + antibacterial)	72	2 = 2.7%

The difference between the death-rates after the antitoxic and the combined immunity amounts to:

$$29.1\% - 2.7\% = 26.4\% \pm 9.5\%. \quad T = 2.8$$

The difference therefore seems to be significant.

A joint summary of the similar third and fourth series results shows:

Kind of Immunization	Number of Animals	Died
None	22	12 = 54.5%
Antibacterial	24	4 = 16.6%

From the two joint summaries the effect of antibacterial immunity is clearly evident: the death rates of antibacterially immunized animals are always smaller than the corresponding control death-rates. The antibacterial immunity alone proved unsatisfactory, but combined with antitoxic immunity gave a powerful protection, significantly greater than the antitoxic immunity alone.

A typespecific immunity could not be clearly demonstrated:

the results of the two series are in this respect not quite in agreement.

2. *Observations on vaccinated persons.*

a) The reaction of one of the two adults inoculated with Anabac was very strong: a large painful edema and fever (38.5° C.) lasting three days. No further complications. The reaction should be referred to antitoxin, because no one of the 20 volunteers vaccinated with bacterial suspension only, showed such a reaction. The second person showed a moderate response. The agglutinin titer with the serum of the vaccinated adults rose for strain C. diphtheriae Nr. 24 from 1 : 8 up to 1 : 64, a month after the inoculation. The antitoxin level rose this time from 0.06 AU/ml. to 0.5 AU/ml. serum.

b) Six adults inoculated with bacterial suspension (type 24) only, showed the following agglutinin titer 30 days after the third injection:

	Agglutinin Titer for the Type						
Before vaccination	24	49	65	214	54 ‡	155	P.W. 8
Range	2– 16 *	4–32 †	8– 32 †	2– 32 †	0– 0 †	0– 2 †	0– 0 †
Average	9	17	22	9	0	0.5	0
After vaccination							
Range	64–128 *	0–32 †	64–128 †	32–128 †	8–64 †	0–16 †	0–32 †
Average	94	8	80	88	26	4	8

* Average from 6 blood samples.
† Average from 4 blood samples.
‡ 0 = No agglutination even in the dilution 1 :2.

We see that immunization with type 24 bacilli gave a significant rise of the 3 other types titers. It is due to the common O antigen, because the cross agglutinations disappear after absorption of the serum with boiled type 24 bacilli. O antibodies have (according to Lautrop) probably no protective power. We could therefore not assume that the vaccination with type 24 bacilli gave protection against the other types.

c) Thirty-nine children, aged 3–13 years, inoculated with Anabac showed following agglutination titers and antitoxin levels, 30 days after the third injection:

	Agglutinin Titer for the Type							Antitoxin Level AU/ml. Serum
Before vaccination .	24	49	65	214	54	155	P.W. 8	
Range	0–128	0–4	0–16	0–16	0–8	0–0	0–2	0–5.0
Average ..	15	0.63	4.73	3.05	0.94	0	0.11	0.3
After vaccination								
Range	0–250	0–4	4–64	4–128	0–64	0–16	0–64	0.13–8.7
Average ..	87	0.21	31. 7	27. 4	13. 5	2.84	19. 6	3.12

All children but one endured the vaccination very well. The one exception, a 12-year-old child, showed fever and a painful edema lasting two days, no further complications.

We see a significant increase in four other agglutinin titers, besides the specific anti 24 titer. Three of them (65, 214, 54) are identical as in the case of adults. The increased titer for Park-Williams Nr. 8 is most probably due to anatoxin, prepared from PW 8 strain cultures. This strain has no antigens in common with strain 24.

Discussion

The mechanism of experimental infection with Corynebacterium diphtheriae is quite different from the infection under natural conditions. The natural infection is accomplished by only a few bacilli which have first to defeat the local defence mechanisms. Phagocytosis and inflammatory fixation of germs are probably the most important of them. Their effectiveness in conditions of a natural infection is most probably much greater than in challenge with massive experimental doses, given immediately into the healthy tissue. Yet, the opsonising of germs depends on the presence of antibacterial antibodies, to some degree even of typespecific antibodies, as we succeeded in demonstrating (Fleck-Kozak), and as mentioned by Orr-Ewing. Therefore, we must assume that the experimentally found effect of antibacterial immunity would be much greater in natural protection.

The mechanism of diphtheria toxin action, its influence on the invasiveness and dissemination of the germ, are up till now insufficiently known. Antitoxin has no direct effect upon C. diphtheriae. Possibly Amies' assumption is correct, that toxin acts

by suppression of an inflammatory local reaction and by inhibiting phagocytosis in this way. Immunity against toxin (active as well as passive) would have a double protective effect: detoxification and making possible the phagocytosis, which would be inhibited by the necrotising action of toxin. This protective effect of antitoxin would be enhanced by the direct antibacterial immunity, which immediately increased the action against the germs. The two kinds of immunity would in this way act synergistically.

It is, however, possible that the two kinds of immunity act in another way, independently, in two different directions. The role of antibacterial antibodies in diphtheria is also evident from the increase of the typespecific agglutinin level during illness, as shown in Table 2.

The typespecific immunity is difficult to demonstrate experimentally. We tried to demonstrate *in vitro* a typespecific inhibition of growth caused by antibacterial sera. The result was quite distinct in some experiments, *e.g.* the addition of typespecific anti-C. Di. Nr. 65 serum to the culture medium inhibited the growth of C. diphtheriae Nr. 65, but not of the other types. In other experiments no clear effect could be noted, perhaps because of different distribution and accessibility of correspondent antigens in different types. The greater importance of K-antigens than O-antigens can be a priori assumed.

For our combined vaccine we used C. diphtheriae type 24, because of its greatest frequency in our country. The observations on inoculated adults and children showed the innocuity of the vaccine as well as a proper effect on the level of antitoxin and antibacterial antibodies. Certainly, the protective power of Anabac has still to be proved on a large series of observations.

The addition of other serotypes to the vaccine or of isolated antigens according to the country type frequency, must be considered. The strain Park-Williams Nr. 8, though an excellent producer of toxin, is at least in our country useless as a supplier of cellular antigens.

Summary

The protective value of antibacterial immunity alone in conditions of an experimental infection of guinea pigs was shown to be rather small. The antitoxic immunity in these conditions

was much more effective, but a combined immunization (antitoxic + antibacterial) gave the most satisfactory results. The coresponding death-rates were in the ratio:

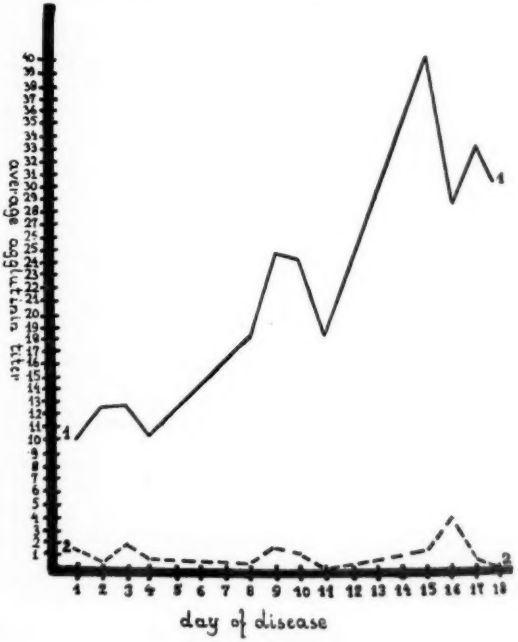
Twenty-nine and one-tenth per cent (antitoxic immunity) : 2.7 per cent (combined immunity). The difference amounts to 26.4 ± 9.5; $T = 2.8$

It is probable that besides the common bacterial antigens, the typespecific antigens play some role.

We can assume that antibacterial immunity in natural conditions of diphtherial infection has a significantly greater protective power than in laboratory experiments. Hence trials were made to enrich the toxoid vaccine with bacterial suspension of

TABLE 2

Agglutinin Level During Diphtheria



1. homotypic strain
2. four heterotypic strains (average levels of 100 cases)

the most common serotype 24. Inoculation of 2 adults and 39 children showed the innocuity of the combined vaccine as well as a proper enhancement of the level of antitoxin and bacterial antibodies.

Therefore, further trial with a combined vaccine (Anabac = anatoxin + bacteria of the serotypes most common in the country) seems to merit consideration.

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* Head of the Department of Microbiology, Mother and Child Institute, Warsaw, Poland, Kasprzaka 17. Accepted for publication August 1, 1957.

SEROTYPES OF CORYNEBACTERIUM DIPHTHERIAE AND THEIR DISTRIBUTION DURING A PROLONGED DIPHTHERIA EPIDEMIC IN POLAND

LUDWIK FLECK,[a] HALINA DUROS-KAWECKA, CECYLIA STANKIEWICZ

The existence of several serologic types of C. diphtheriae was established several years ago. During the last decade Hewitt (1947), Ferris (1950), Lautrop (1950), Oeding (1950), Cummins (1954), have investigated this subject. In some details the results were contradictory, but certain observations have been made consistently.

The classification in serotypes does not correspond to the classification in toxigenic types of gravis, mitis and intermedius, nor does a correlation exist between the serologic type and virulence or toxicity. In cases originating from a single, common source, the same type of diphtheria organism appears in the infected individuals. In each diphtheria epidemic one type is more frequently found, comprising 60–80 per cent of all isolated strains, the other types being rather rare.

More recent investigations (Wong and T'Ung, 1938–1940) indicate that C. diphtheriae contains more specific protein antigens characteristic for a single type and less specific carbohydrate and lipid antigens common for several types. Lautrop (1950), Oeding (1950) and Cummins (1954) stated that the thermolabile protein antigen is located near the surface of the diphtheria bacillus, while the thermostable carbohydrate antigen lies deeper in the cytoplasm. The surface antigen has been denoted by Lautrop with the symbol K, the deeper antigen the symbol O. The O antigen is partially common for C. diphtheriae, C. hofmanni and C. ovis. There are several O antigens and K antigens which in various combinations contribute to the antigenic structure of a serotype.

Methods and results

The purpose of our investigations was to apply the typing of C. diphtheriae to the epidemiology of the disease in correlation

with the appearance and distribution of the various serologic types of the organism. About 2000 strains from diphtheria patients and carriers in the whole country were collected during the past four years of the study. The typing consisted, in principle, in agglutination tests performed in small tubes or capillaries containing 3 per cent sodium chloride. In using about one hundred agglutinating sera from immunized rabbits, cross agglutinations as well as cross absorptions were made with suspensions of the particular diphtheria organism. Fourteen distinct serological types were tentatively labeled with the numbers of our standard strains as follows: *24, 25, 49, 54, 65, 155, 214, 1296, 2010, 2094, 1772, 2126, 2083, 2055*. In checking with the 14 standard antisera, about 80 per cent of the strains were identified, while the remaining 20 per cent could not be classified within any of the 14 types. It was found that the O antigen, common for almost all serotypes, is responsible for most of the overlapping cross reactions. Some strains, like C. hofmanni, C. xerosis and C. equi have one O antigen in common with C. diphtheriae, besides, there are other O antigens of narrower specificity, characteristic for a single or for several types. Sometimes the K antigens inhibit partially or completely the O agglutination. For this reason one anti-serum (anti–*24*) agglutinates bacteria of a different type (25), but the anti–*25* serum will not agglutinate bacteria of type *24*. Each serotype contains a specific K antigen, morever some types have secondary K antigens with other types in common such as type *25* with type *49*.

The distribution of the serotypes in Poland is as follows: Type *24* is the most frequent, the average frequency in the years 1953–1957 for the whole country amounts to 62.5 per cent and varies in single districts from 38–80 per cent. This type is identical with type II of Hewitt (1947) as established by us by comparison with standard strains received from Britain[b]. The second in frequency is type *49* (country's average 2.8 per cent, in single districts 0–5.1 per cent). The frequency of other types was 0.05 per cent to 2.3 per cent for the whole country and up to 6.15 per cent for single districts. The frequency in the Warsaw district was similar to the country's average, whereas the prevalence in the frontier districts of Bialystok or Szczecin was very different.

Among 20 strains received from Hungary, 16 were of serologic type *24* (80 per cent), the remainder could not be classified.

Among 28 strains received from Czechoslovakia, 12 (42 per cent) were of the type *24*, the remainder could not be identified with our sera. It is interesting to note that in 1936 Robinson and Peeney examined 15 strains from Poland and found all of them type II corresponding to our type *24*.

Table 1 illustrates the fluctuations in serotype frequencies based on typing in the years 1953–1955, 1956 and 1957. The frequency of the main type *24* seems to decrease during these years; on the contrary, the number of the unclassified rare or new strains increases. One type *(1772)* disappeared after 1955, while other types, previously absent, appeared in 1956 *(2055)* and in 1957 *(2126)*. Striking are the fluctuations of type *49:* 2.5 per cent in 1953–55, 9.7 per cent in 1956 and back to 2.4 per cent in 1957. The increase as well as the decrease are statistically significant.

Table 2 shows the increase taking place particularly in 3 districts: Katowice, Poznań and Warszawa, namely from 2.5 per cent to 13.1 per cent. The difference (10.6 — 2.16) is significant.

No strains of the type *49* were found in any of the three districts in 1955, but they were present in small numbers in some remote areas. We found only eight strains of type *49* (1.72 per cent of the total) in 1955 as compared with 9.7 per cent in 1956. Detailed records indicate that the sudden increase of type *49* frequency occurred primarily in Poznan and Katowice, between January and July 1956 and lasted until 1957. Besides the 21 strains of type *49*, we found 16 atypical strains in Poznań among 119 examined. These atypical strains were weakly agglutinated with the standard anti–*49* serum and reacted also weakly with other standard sera. These strains are placed among the unclassified groups (Table 2).

Table 3 shows the distribution of type *49* in three districts and of other strains weakly agglutinated with anti–*49* serum. It can be seen that the number of the atypical strains grows simultaneously with the increase in number of type *49*.

Discussion

The results of our investigations indicate that even when a diphtheria epidemic is limited to a small area or to a single town, a multitude of certain serotypes of C. diphtheriae is involved. In Poland, one certain type *(24)* dominated quantitatively and this

TABLE 1

The distribution of serotypes of C. diphtheriae in Poland in the years 1953–1957

Years	Number of Exam. Strains	Identification Numbers of Serotypes: *24*	*25*	*49*	*54*	*65*	*155*	*214*	*1296*	*2094*	*2010*	*1772*	*2126*	*2083*	*2055*	Unclassified
1953–1955 ..	1132	747	4	28	10	8	6	41	19	0	0	39	0	0	0	288
		66.1%	0.3%	2.5%	0.9%	0.7%	0.5%	3.6%	1.7%			3.4%				20.1%
1956 ...	506	291	2	49	1	12	4	8	14	1	1	0	0	1	15	107
		57.5%	0.4%	9.7%	0.2%	2.4%	0.8%	1.6%	2.8%	0.2%	0.2 %			0.2%	2.9%	21.1%
1957 ...	82	35	0	2	2	0	4	0	0	1	0	0	1	1	1	35
			42.7%		2.4%	2.4%		4.8%		1.2%			1.2 %	1.2%	1.2%	42.7%
Total ..	1720	1075	6	79	13	20	14	49	33	2	1	39	1	2	16	370
		62.5%	0.3%	4.6%	0.7%	1.2%	0.8%	2.8%	1.9%	0.1%	0.05%	2.3%	0.05%	0.1%	0.9%	21.5%

TABLE 2

The frequency of 7 serotypes in the years 1953–1955, and 1956 in the Districts: Katowice, Poznan, Warszawa

Serotypes	Years 1953, 1954, 1955: *24*	*25*	*49*	*54*	*65*	*155*	*214*	Unclassified	Total	Year 1956: *24*	*25*	*49*	*54*	*65*	*155*	*214*	Unclassified	Total
Katowice	33	0	6	0	0	1	5	23	68	6	0	10	2	0	0	0	20	36
	48.5%		8.8%			1.5%	7.5%	33.8%		16.6%		27.7%					55.5%	
Poznan	50	0	0	0	0	0	1	19	78	53	1	21	1	3	0	4	36	119
	74.3%						1.3%	24.3%		44.5%	0.8%	17.6%	0.8%	2.5%		3.3%	30.2%	
Warszawa	50	0	0	4	0	0	2	36	92	104	1	9	0	3	0	2	30	149
	54.3%			4.3%			2.1%	39.1%		69.8%	0.7%	6%		2%		1.3%	20.1%	
Total in 3 districts	141	0	6	4	0	1	8	78	238	163	2	40	1	6	0	6	86	304
	59.2%		2.5%	1.7%		0.4%	3.4%	32.8%		53.6%	0.6%	13.1%	0.3%	2%		2%	28.3%	
In remaining districts	608	4	22	6	8	5	33	208	894	128	0	9.0	0	6	4	2	53	202
	68%	0.4%	2.4%	0.7%	0.9%	0.5%	3.7%	23.3%		63.3%		4.4%		3%	2%	1%	26.2%	

In remaining districts	608	4	22	6	8	5	33	208	894	128	0	9.0	0	6	4	2	53	202
	68%	0.4%	2.4%	0.7%	0.9%	0.5%	3.7%	23.3%		53.3%		4.4%		3%	2%	1%	26.2%	

TABLE 3

The frequency of typical serotype 49 and atypical strains in the Districts: Katowice, Poznań, Warszawa

Number of Strains and Frequency in Percentages

District	1953			1954			1955			1956		
	Examined	Typical 49	Atypical	Examined	Typical 49	Atypical	Examined	Typical 49	Atypical	Examined	Typical 49	Atypical
Katowice	...	..	..	42	6	1	26	0	2	36	10	4
				100%	14.3%	2.4%	100%		7.7%	100%	27.7%	11.1%
Poznan	15	0	1	59	0	0	4	0	0	119	21	16
	100%		6.7%	100%			100%			100%	17.6%	13.4%
Warszawa	37	0	2	19	0	4	36	0	0	149	9	3
	100%		5.4%	100%		21%	100%			100%	6%	2%

strain is probably common in many other countries. The prevalence of other, less frequent types differed greatly in single districts. Remarkable fluctuations in type distribution and frequency could be observed as well as the appearance and disappearance of certain types was recorded. This applies particularly to a sudden appearance of type *49* in certain districts.

Parallel with the increase in number of the typical strain *49*, similar atypical strains were found which disappeared simultaneously with the typical strain. Two possibilities can be considered, namely, the possible importation of type *49* from abroad during the International Fair in Poznań (June, 1956), furthermore, the possibility of a local antigenic variation which produced a new, highly contagious type is to be considered. These varieties could be agglutinated only up to 25 per cent of the titer of the standard anti–*49* serum and with no other standard antiserum. Nevertheless, we succeeded in complete absorption of anti-*49* antibodies from the standard serum by means of the atypical variants. Whatever the explanation of these phenomena may be, the observed type fluctuations may contribute to a better understanding of the dynamics of an epidemic.

Summary

During a prolonged diphtheria epidemic in Poland (1953–1957) fourteen serotypes of C. diphtheriae were differentiated. One type *(24)* dominated quantitatively up to 60 per cent of other strains. A variable type distribution was observed in various districts as well as a perpetual fluctuation of type frequency. During the period of observation some types disappeared while new types made their appearance. The appearance of type *49* in three districts was accompanied by atypical weakly agglutinable strains. The analogy with inagglutinable phage resistant strains and a possibility of typogenetic processes is considered.

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[a] Head of the Department of Microbiology, Mother and Child Institute, Warsaw, Poland, Kasprzaka 17. Accepted for publication August 1, 1957.

[b] Hewitt's types I, III, IV, V have no serologic equals with our 14 types.

TOXIC VAPOR INHALATION AND SERUM ENZYME LEVELS

HAROLD D. FRANKL,[a] PAUL L. GAERTNER,[a] LOUIS C. KOSSUTH,[b] AND LAWRENCE J. MILCH[a]

Molander *et al.* (5,6,7) demonstrated that serum glutamic oxalacetic transaminase (SGOT) served as a sensitive index of hepatocellular damage. Wroblewski and LaDue (9) reported on two patients that had been exposed to the fumes of carbon tetrachloride, displayed the characteristic symptoms and signs of CCl_4 poisoning and showed SGOT levels in excess of 25,000 and 12,000 units per milliliter, respectively (One unit has been defined by Karmen as the amount of enzyme which will cause a unit drop in optical density per minute under conditions of the test (4)). These observations suggested that routine, repeated, serum enzyme assays might prove useful in the early identification of extraordinarily sensitive individuals chronically exposed, in the course of their occupations, to vapor concentrations of industrial solvents presumably below toxic limits. Thus, exposure could be discontinued before gross signs of poisoning appear.

The threshold limit value for CCl_4 is 25 ppm. by volume (3). This value represents the allowable maximum average concentration for an 8-hour per day chronic exposure. However, no irreversible or permanent injury has been reported with concentrations <100 ppm. It was decided, therefore, to subject rabbits to single 5-hour exposures of CCl_4 in concentrations of 120 ppm. and less. Each animal served as its own control for serial determinations of serum GOT, glutamic pyruvic transaminase (SGPT), lactic dehydrogenase (SLDH) and malic dehydrogenase (SMDH).

Method

Carbon tetrachloride vapor was delivered to the animal chamber by passing a metered quantity of air over a wick suspended in carbon tetrachloride. This provided thorough mixing of air and vapor prior to delivery to the cage. Chemical assays were done by the method of Rogers and Kay (8). For each assay 300 cc. of air were withdrawn from an outlet centrally placed on the cage. Three determinations were made during each 5-hour animal exposure.

Conversion to ppm. in air was accomplished by use of the factor 1 ppm./0.0063 mg. CCl_4/L. air (1) at 760 mm. Hg at 25° C.

Thirty albino rabbits of the New Zealand strain were used—25 being exposed to CCl_4 vapor and 5 serving as controls. An animal was placed in a lucite cage 26 × 42 ×28 cm. for the 5-hour exposure period. Air flow was maintained at 10 L./min. (± 2 L./min.). The cage temperature was equal to room temperature at all times (27° C.). Controls were treated in the same manner as the CCl_4 rabbits except for the omission of the CCl_4 wick in the system.

Enzyme determinations were made on samples obtained by incising the marginal ear vein of the rabbit. Samples were taken just prior to exposure, immediately after exposure (post 5-hour), and each afternoon thereafter. SGOT and SGPT activities were measured according to the method of Karmen, Wroblewski, and LaDue (4). SMDH and SLDH activities were determined by the method described by Albaum (2).

Discussion

The changes in enzyme activity are shown in figures I through IV. It is of considerable interest that while every animal demon-

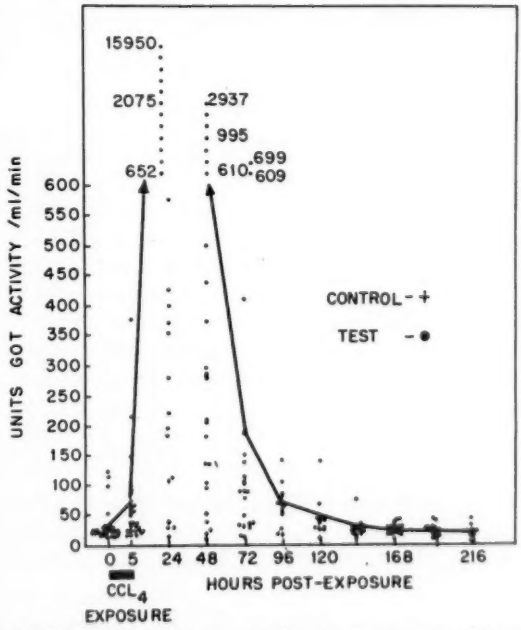

FIG. I. Serial SGOT levels prior to and following exposure to CCL_4 vapor. The solid line represents the aveage of test values.

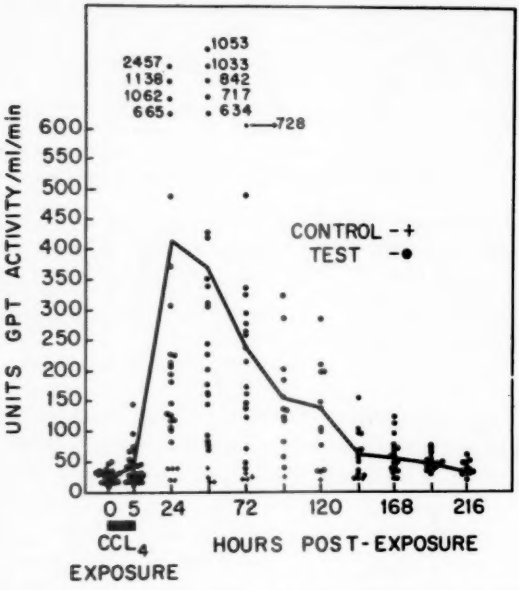

FIG. II. Serial SGPT levels prior to and following exposure to CCl_4 vapor. The solid line represents the average of test values.

strated a significant response to the insult, no consistent correlation between vapor concentration and enzyme activity level was apparent. In the narrow range of concentration utilized, certain animals exhibited striking elevations of enzyme activity at relatively low concentrations and others showed relatively small changes after exposure to concentrations of over 100 ppm. Table 1 has the peak changes of SGOT activity tabulated in reference to the CCl_4 concentration.

Summary

1. Twenty-five New Zealand white rabbits were exposed to carbon tetrachloride vapor for a 5-hour period. Vapor concentrations utilized ranged from 30 to 120 ppm. Five control animals were also run.

2. Serum enzyme activities of glutamic oxalacetic transaminase, glutamic pyruvic transaminase, lactic dehydrogenase and

malic dehydrogenase were measured prior to CCl_4 exposure, at the end of exposure, and at 24-hour intervals thereafter.

3. Significant increases in serum enzyme activity occurred in all animals and for all enzymes tested. No increases were noted in the control animals.

TABLE 1

Individual values for per cent SGOT activity increases at 24 hours are segregated according to the CCl_4 vapor concentrations which initiated them

Parts per Million					
30–60	60–70	70–80	80–90	90–100	100–120
1,890	435	71	375	2,400	31,600
1,016	3,470	417	3,920	950	1,420
7,570	63,700	20,900	18,370	11,600	4,970
1,055	5,220	2,050	60,400		7,890
					4,120

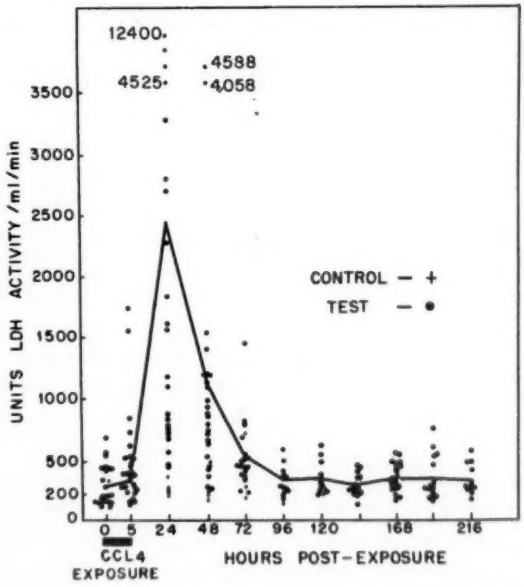

FIG. III. Serial LDH levels prior to and following exposure to CCl_4 vapor. The solid line represents the average of test values.

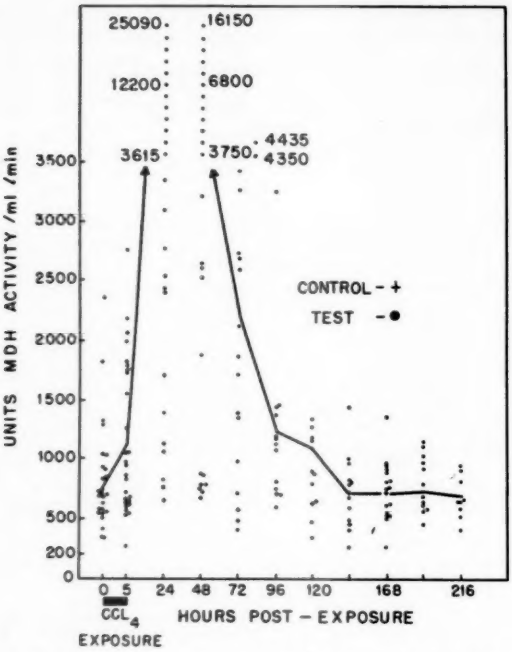

FIG. IV. Serial MDH levels prior to and following exposure to CCl_4 vapor. The solid line represents the average of test values.

4. Peak values were seen in 24 hours with SGOT, SLDH, and SMDH. These returned to essentially normal levels in 96 hours. SGPT activity exhibited peak levels throughout the 24–72-hour period.

5. Individual variation was marked and the degree of enzyme activity change did not correlate significantly with the concentration of CCl_4 vapor in the range 30–120 ppm.

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[a] Department of Pharmacology-Biochemistry School of Aviation Medicine, USAF Randolph Air Force Base, Texas.

[b] Present address: Deputy Surgeon, Headquarters Air Defense Command. Ent Air Force Base, Colorado, Accepted for publication August 2, 1957.

THE HISTORY OF GOITER IN THE INCA EMPIRE: PERU, CHILE AND THE ARGENTINE REPUBLIC[a]

Its Significance for the Etiology of the Disease

Isidor Greenwald[b]

I. Introduction

In contrast to the prolonged controversy over the pre-Columbian presence of syphilis in the Americas, the question of whether or not goiter was endemic has attracted little attention. This is the more remarkable since the answer is of great importance in a consideration of the cause of endemic goiter.

Since this disorder is generally believed to be due to the environment, supposed not to have changed, it has been assumed that goiter must have ante-dated Columbus on this continent. An excellent example of this attitude may be found in the book by Stone (46): "In certain areas, goiters and urinary calculi were endemic as they are today in these regions among both their white and red inhabitants." Inquiry as to the sources for this statement elicited the reply: "Goiter was not noted by the early explorers or pioneers" (47).

I have selected for study the territories of the former Inca Empire. They are particularly well-suited for this purpose because extant contemporary accounts of the civilization of the inhabitants are as satisfactory as those for any other part of the Americas, because we have the reports of numerous visitors over a period of more than three centuries and because we have detailed scientific and statistical information for more recent periods.

II. The Inca Empire

To a great extent, the belief in the endemicity of goiter in the Inca Empire is due to a grotesque error in translation. In 1609, Garcilasso de la Vega published "Los comentarios reales de los Incas" (18). This was translated by Sir Paul Rycaut (41), who wrote:

"From *Casamarquilla* the Inca proceeded to another People called *Papamarca*, from the *Papas* or Dewlaps which are great bunches that hang from their throats."

This statement was quoted by Barton in his memoir (6) and from him by many others.

What Garcilasso de la Vega wrote was:

"De Cassamarquilla paso a otro pueblo principal, llamado Papamarca, que quiere decir pueblo de papas, porque son muy grandes que alli se dan." (From Cassamarquilla, he passed to another village called Papamarca, which means village of potatoes because they grow very large there.)

Nevertheless, Rycaut's gross error has persisted in the literature. As recently as 1951, it was used by Lastres (27) to support his view that goiter was endemic in pre-Hispanic Peru.[e]

At about the same time as Garcilasso de la Vega compiled his Commentaries, Guzman Poma de Ayula wrote a lengthy work describing the inhabitants of Peru (24). He accompanied the text with hundreds of drawings. There is no mention of goiter in the text nor any indication of goiter in the drawings. If there had been, at the time, anything resembling the huge goiters later so common in Peru, Bolivia and the Argentine Republic, it is almost certain that they would have been described and depicted.

Among the works of the ancient Peruvians that have survived are ceramic figures showing a great variety of diseases and deformities. There are a few statements in the literature that some of these show goiters but there is only one published photograph and only one statement as to where this figure, or any other, was seen (Velez Lopez, 55). In the picture, the exophthalmos is pronounced but the goiter is not visible. Even if this ceramic were a representation of exophthalmic goiter, it would not be evidence of the *endemicity* of goiter.

It may be, as Monge (34) has stated, that no ceramics showing goiter could be expected because most of the ceramics have been found in districts in which goiter is not common. One might take exception even to this for ceramics have been found near Cuzco and goiter has been prevalent there since 1638.

There has been considerable confusion between parotiditis and goiter throughout Spanish America (and in Spain as well) in the sixteenth, seventeenth and eighteenth centuries and even well into the nineteenth. The same Spanish word "paperas" (now used for parotiditis) and the same Indian word "coto" were used for both disorders.

The earliest dictionaries of the Quicha language (dominant in the Inca Empire) date from 1608, 1614 and 1619, respectively. The first (21) gives the Spanish equivalent of "ccoto" as "monton" (heap, pile). It also gives the Indian equivalent of "papera" as ccoto. The second (4) defined "coto" as "papera." The third (53) listed "ccoto" as "monton" and "ckoto" as "papera." The word "coto" seems also to have been used to mean mountain as in "Cotopaxi."

There can be no question but that parotiditis was seen not long after the Spaniards came and was very severe. The earliest reference that I have found is the statement by Toribio Polo (52). According to this, the Anales de Cuzco recorded

"Durante el mes de Abril (1585) corría en esta ciudad una peste de tabardillo y paperas que, aunque duró poco tiempo, dió bastante cuidado." (During the month of April 1585, there ran through this city an epidemic of typhus (?) and mumps, which, though lasting only a short time, caused much fear.)

In his history of the Francisican missions, Izaguirre (25) wrote of the Panatahuas Indians of about 1630:

"Tambien se había reducido el número de los paysanos, a causa de las enfermedades de serampion, viruela, paperas y otras. Se morian ademas casi todos las criaturas en sus primeros años." (Also, the number of country-folk was reduced because of measles, smallpox, mumps and other diseases. Almost all the children died in their first years.)

The word "papo", which like the German "Kropf" and the Italian "gozzo" means the crop of a bird, was employed for goiter in the Asturias (Spain) in the sixteenth century (Daza Chacon, 12). However, such use seems to have been strictly localized for a long time. I have not found the word in any of a number of medical works published from the fifteenth century to the nineteenth. The various dictionaries published by the Academia Española give no such meaning in the original of 1737 nor in any of the condensations as late as 1914. The first definition "Papo = (3) Nombre vulgar *del bocio* en las regiones donde es endemico" (the common word for goiter in regions where this is endemic) appeared in 1925.

There are two early instances of the use of this word in the literature relating to South America. In 1586, de Acuna wrote (13):

"Al trece capitulo dijo que este pueblo de Cotoguasi en lengua de indios, se dice coto como papo y que se llama ansi por estar un alto de un mogotillo de un cerro." (In the thirteenth chapter I state that this village of Cotoguasi in the language of the Indians, that is to say "Coto" like "papo" and which is so called because it is on a height on a rock on a mountain.)

To this, the editor, Jiminez de la Espada, added a footnote:

"El otro componente de la palabra, *guaci* o *huasi* significa casa; de donde casa del papo, o mejor casa del bulto, del monton, o del cerrilo, si la primera parte es realemente corrupcion de *coto*, y no otra causa." (The other component of the word, *guaci* or *huasi*, means house, from which house of "papo" or, better, house of a protuberance, pile or hill, if the first part is really corruption of *coto* and not something else.)

A few years later, Lizarraga (29) wrote:

"De los valles y pueblos desde Clisa a Mizque . . . tiene otra plaga, y es se crian en los indios como en los españoles, papos, que aca llamas cotos, en las gargantas. Yo he visto hijos de españoles nacer con ellos. El remedio experimentado es atarse a la garganta uno o dos cabezas de viboras y con esta y disvelten." (In the valleys and villages from Clisa to Mizque (now in Bolivia) . . . there is another plague, which creates in Indians and Spaniards "papos", which are here called "cotos", in the throat. I have seen children of Spaniards born with them. The tested remedy is to attach to the neck the heads of one or two vipers and thus dissolve them.)

The context seems to make it quite clear that De Acuna used "papo" only as meaning a lump or protuberance and it is probable that Lizarraga employed it in the same sense. If he had meant "goiter" and had believed that his readers would so understand "papo", the phrase "en las gargantas" would have been superfluous.

Moreover, while the sentence "Yo he visto hijos de españoles nacer con ellos" might seem to refer to neonatal goiter, the only case of "papos" described was in a *man*, who was treated and cured in the manner mentioned above. The treatment suggests that the disorder was parotiditis or a swelling of the lymph glands.

Even if either, or both, of these authors meant "goiter," it must be remembered that they wrote fifty and seventy years, respectively, after the Conquest. Elsewhere, in fifty years, goiter has appeared, become truly endemic and *disappeared!* (22).

III. PERU

In 1638, one hundred years after the Conquest, Calancha (9) wrote:

"Solo en las tierras del Cuzco y Chuquisaca hay alguna aqua que a tal, o tal persona crian hinchazones en las gargantas, que llaman cotos." (Only in the lands of Cuzco and of Chuquisaca (now Sucre, Bolivia) is there water which produces swellings in the throat called cotos.)

These swellings were probably goiters and this statement about Cuzco is the first mention of goiter in the territory of present-day Peru.

Before 1638, and after, there were several accounts of Peru, describing localities in detail. One of these (54) praised Yucay as "a bit of Paradise" (p. 601) and stated, of Huanuco, "the city is very healthy." (p. 488). As late as 1758, there was published a long and detailed description of all of the provinces in the archdiocese of Lima (8). "Cotos" were said to be prevalent in two districts in what is now Bolivia and also in Salta (now in the Argentine Republic) but there was no mention of their presence anywhere in what is now Peru.

However, only twenty years later (*e.g.*, 1778), goiters were common at Huanuco (see above) but were not mentioned as occurring elsewhere, notably, not at Pozuzo (see below) (40). In 1792, a letter from Huancavelica concerned a new specific of undisclosed nature for curing cotos (16). By 1814, goiter was, apparently, still quite frequent at Huanuco (35) and, in 1825, goiters were common in other northern provinces "particularly in the neighbourhood of Huacaibamba" (45). A few years later, the disorder was said to be prevalent in all the interior valleys of Peru (44). Association with deaf-mutism was noted but the presence of cretinism was explicity denied. By 1867, goiters were seen at Pozuzo (see above) (50). Another report (1), of the same year, stated that these goiters were accompanied by cretinism but that both had diminished recently, since iodine had been used. (It is noteworthy that the population of Pozuzo consisted chiefly of Tyroleans and their immediate descendants.)

In 1886, goiter and cretinism were common in many places, including Yucay ("a bit of Paradise" early in the seventeenth century). Neither goiter nor cretinism was as common as they had been forty years earlier. The change was believed to be due

to improved economic conditions, leading to an increased consumption of meat (30).

TABLE 1

PERU

Number of persons with obvious goiters, of deaf mutes and of "dementes," per 100,000 of total population in the various departments of Peru in 1940.* (42)

Department	Goiters	Deaf Mutes	"Dementes"	Department	Goiters	Deaf Mutes	"Dementes"*
Amazonas	2879	843	200	La Libertad	638	446	110
Ancash	1436	649	168	Lambayeque	5	86	77
Apurimac	988	375	145	Lima	13	59	247
Arequipa	68	132	109	Loreto	21	78	38
Ayacucho	428	281	146	Madre de Dios	81	40	..
Cajamarca	363	366	159	Moquegua	38	173	132
Callao	9	30	27	Piura	93	214	135
Cuzco	492	316	105	Puno	124	204	107
Huancavelica	877	610	303	San Martin	246	161	130
Huanuco	1996	669	134	Tacna	8	105	94
Ica	7	72	68	Tumbes	16	136	132
Junin	509	397	112	Total	473	300	145

* The only classification of defects was Bocio o coto, Sordo-mudos, ciegos, invalidos, dementes, verruga and uta. (37).

As may be seen from table 1, goiter and deaf-mutism were common in Peru in 1940 (37, 42). According to Salazar (42), Guzman Barron, in 1920, had found some places in the Department of Ancash in which every person had a goiter, whereas in other places in the same Department only five per cent of the population were so afflicted.

Of the province of Pachitea, in the Department of Huanuco in 1950, Salazar wrote: (p. 47)

"Panao, Chaella, Molinos, libres de bocio pero se encuentra en las zonas boscosas del Pozuzo, cretinismos notables en raza blanca de origen tiroles (immigrantes)." (Panao, Chaella, Molinos are free from goiter but in the wooded zones of Pozuzo, pronounced cretinism in the white race of Tyrolean origin (immigrants) is encountered.)

The material in this section shows that the earliest mention of goiter in Peru dates from a century after the Conquest and explicitly states that the disorder was present in only one locality (Cuzco) in Peru. Successive accounts by many observers show its gradual spread over a large part of the country, with occasional diminution in certain places. Attention is directed to the

large number of deaf-mutes in regions in which goiters were most common. This relation will be discussed in another paper.

IV. Chile

This country seems to have been free from goiter until quite recently. None of the descriptions of the Spanish possessions including Chile, written during the sixteenth, seventeenth and eighteenth centuries referred to the existence of goiter in Chile although several noted its presence in various parts of what are now Peru, Bolivia and the Argentine Republic. The first mention of goiter in Chile that has been found was written in 1814 and stated that the disorder was present but was not common; not of the same order of prevalence as at Huanuco (Peru) or Mendoza (Argentine Republic) (35).

Of conditions in 1820 or 1821, we have two reports. One (10) reads:

"This malady appears to prevail all down the eastern slope of the Andes; but on the western, or Chile, side, it is comparatively rare."

The other stated:

"The inhabitants of Santiago, however, do not exhibit the same enlargement of the neck and head as the Mendozines; the goitre . . . is neither so common nor, except perhaps in a few cases, so protuberant as at Mendoza. I observed it in women inhabiting within a few leagues of Santiago, and in the midst of irrigated grounds, but nowhere else in Chile during a journey of above a thousand miles, along both the sea shore and the foot of the Andes" (43).

About thirty years later (1850), two independent accounts stated that the people of Santiago regarded their goiters as a recent affliction.

"I was informed that the *goitre* was unknown in Chile until some twenty years ago, when it made its appearance simultaneously with the introduction of poplars from Mendoza" (48). "Some say that the goitre was not known until the Maypu emptied part of its muddy stream into the Mapacho; others assert that it came with the poplars from Mendoza" (20).

By this time, goiter was also seen in other places. In fact, Lafargue (26) is credited with having stated that it was present in

all places more than fifteen leagues from the coast. Specifically, Gillis wrote of Talca, which is rather nearer to the ocean, that "Goitre, though known, is not so prevalent as farther to the north" (20).

In 1862, Guyon (23) wrote that goiter was as common in Santiago as at Mendoza (perhaps most notorious for its goiters of any district in the former Inca empire).

In 1778, the vicinity of Concepcion had been called a terrestrial paradise (40). Some fifty years later, Poeppig (38) stated that goiter existed only in traces. However, after another century, Suazo Figueroa (49) found goiters in 2.3 per cent of 1,085 routine autopsies, or in 3.5 per cent of those upon women. During the 100 or 150 years, goiter had progressed southward from Santiago to Concepcion.

There were changes nearer Santiago. The first report of goiter in Valparaiso was that of Born (7), in 1892, who saw many goiters in the hospitals of Valparaiso. In 1951, Feferholtz and Ortiz (15) wrote of Boco, a village near Quillota, northeast of Valparaiso and about twenty-five miles from the ocean, that goiter was so prevalent as to give one the impression that one had been transported to a village in Switzerland.

Not until 1930 was there mention of cretinism in Chile. This was at Teno (3). The presence of cretinism in Chile had been expressly denied by Poeppig (38) a century earlier and by Born (7) as late as 1892.

The late development of goiter in Chile and its comparatively mild effects are also indicated by the small number of deaf-mutes recorded in successive censuses (Table 2). Whereas, in 1862, it was said that goiter was as common at Santiago as at Mendoza (23), the number of deaf-mutes in the province of Santiago was only 66 per 100,000 in 1865 and 81 in 1875, as compared with 1,321 in 1869 in the department of Mendoza. Guyon's statement of 1862 may not have been accurate but it could scarcely have been so completely erroneous as the ratio of 66 or 81 to 1,321 might indicate. As Table 2 indicates, the incidence of deaf-mutism was not regularly higher in the goitrous provinces than in those quite free from goiter and in many instances, it was appreciably lower.

In Chile, there has been no separate enumeration of people with goiters. However, there are figures for the number of per-

TABLE 2

Number of deaf mutes per 100,000 of total population in Chile and in four provinces thereof. Calculated by the author from the data in the census.

Year	Chile	Valparaiso	Santiago	Concepcion	Talca
1854	9	9	12	8	2
1865	55	39	66	69	26
1875	55	33	81	57	51
1885	67	52	78	58	81
1907	72	107	89	68	31
1920*	99	82	92	76	84

* Of the remaining 20 provinces, only 5 had rates as low as 76. Twelve had rates of 99, or more; one Arauco, 186. These were probably not due to disorders of the thyroid for, in 1922, the four provinces listed above furnished all but four of the cases of diseases of the thyroid treated in hospitals and, in 1923, all but three. See also Table 3.

TABLE 3

*Annual admissions to hospitals because of diseases of the thyroid in Chile and in four provinces thereof. Calculated from data in Anuario estadistica for each year.**

Year	Chile	Valparaiso	Santiago	Concepcion	Talca
1922–7	56	9	40	1	0.5
1928–30	164		88	10	2
1931–6†	252		156	16	3
1937–9†	302	47	173	17	3
1940†	380	54	202	22	7
1941†	852	74	357	82	26
1942†	494	75	248	26	18
1943†‡	504	84	270	33	12
1944†‡	544	76	320	35	10
1945†‡	506	61	311	25	11
1946†‡	471	63	271	20	5
1947‡	527	48	323	38	9
1948‡	584	57	335	40	10
1949‡	592	55	356	35	15
1950§			150		
1951§	277		110		

* Blank spaces indicate that no information is available.
† Include diseases of parathyroids, but not tetany.
‡ Include small number of deaths, not in hospitals.
§ *New* cases seen by state physicians, in or out of hospitals.

sons admitted to the hospitals because of diseases of the thyroid (Table 3). The data, from year to year, are not strictly comparable because of differences in classification. However, they do seem to indicate a pronounced, and general, increase beginning in the 1920's and very marked in 1941. After that, there was a decline. Later the number remained fairly constant, in proportion to the total population, to 1949, the last year for which such data are available.

In this section, the late appearance and mild nature of goiter in Chile have been described. The contrast with conditions in Peru (preceding section) and in the Argentine Republic (following section) are marked.

V. The Argentine Republic

As Table 4 shows, goiter has been most prevalent in the northern and northwestern departments. In these, it had, for years, attained a frequency that has been exceeded in but few regions, even in the Alps.

However, the earliest report of goiter did not come from those sections but from much to the south and east thereof. In 1658, Acarette de Biscay visited Santiago del Estero and wrote:

"The greatest part of the women are handsome enough but have generally a kind of swelling in the throat which they call "coto" (2).

TABLE 4

ARGENTINE REPUBLIC

Number of persons with obvious goiters and number of idiots, including cretins, per 100,000 of population in 1869 and 1895. Number of deaf-mutes, per 100,000 in 1869, 1895 and 1914.† (By 1914, the number of goiters had diminished so greatly that no separate classification was believed necessary.)*

Department	Bocios*		Idiotas*		Sordo-mutos†		
	1869	1895	1869	1895	1869	1895	1914
Capital	20	11	64	37	118	54	67
Buenos Aires ..	25	21	139	43	169	56	61
Santa Fe	20	21	90	27	170	40	47
Entre Rios	51	39	138	56	159	60	81
Corrientes	187	44	252	56	183	114	87
Cordoba	64	24	177	55	297	98	77
San Luis	109	187	321	72	504	152	97
Santiago del Estero	35	44	80	52	166	133	88
Mendoza	2712	1829	508	191	1321	684	252
San Juan	106	31	230	77	348	73	55
Rioja	648	463	320	96	691	364	246
Catamarca	279	872	309	120	354	372	176
Tucuman	552	211	369	90	491	217	166
Salta	1127	538	912	239	1287	769	700
Jujuy	1325	1147	854	288	1946	1160	673

* From the second census.
† From the third census.

From Santiago del Estero, Acarette de Biscay travelled north through places that later greatly exceeded Santiago in the prevalence of goiter but he made no mention of its presence in any of these. However, a few years later (1673), goiter seems to have been fairly common in at least part of this region for it was said that many of the streams gave "cotos" to those who drank from them (51).

A more precise localization may be inferred from the report by Cosme Bueno (8). About 1750, he mentioned goiters in two places in what is now Bolivia but only in Salta in the present Argentine Republic.

The first mention of goiter in, or near, Mendoza that I have found is the statement by Perinetti, Yaciofano, Staneloni and Nora:

"In 1783, Don Nicolas de la Cruz y Bahamonde, Count of Maule, while travelling along the high road from Mendoza to Buenos Aires, found, at the Corocorto post-house (on the Mendoza-San Luis boundary) that "the man in charge, originally from Malta, married and with three children, had, as well as all his family, goiter in the neck from drinking the rather salty water which runs in a nearby rivulet" (36).

It has not been possible to obtain information as to the origin of this statement. However, since de la Cruz y Bahamonde came to this post-house *after* he had travelled from Talca to Concepcion and from there to Santiago (Chile) and Mendoza (11) it would appear that goiter, if present at all in these places, was not particularly noticeable. In fact, it is quite possible that this man from Malta and his family introduced goiter into this region.

At any rate, while early reports (54, 8), as well as those of visitors in 1698 (14) and in 1795 (5) made no mention of goiter at Mendoza, those who came some forty years after de la Cruz y Bahamonde remarked on its prevalence (33, 10, 43). However, the presence of *cretinism* was expressly denied by Miers (33) and was not noted by the others, nor by MacRae (31) nor by Strain (48), some thirty years later, nor by Martin de Moussy (32), late in the 1850's, who did remark on the occurrence of cretinism elsewhere in the Argentine (Jujuy and the valley of Famatina in La Rioja).

However, though cretinism was absent, or infrequent, at Men-

doza in 1819, deaf-mutism seems to have been common. Caldcleugh (10, vol. 1, p. 289) wrote:

"One woman of my acquaintance, herself a goteira, had five children similarly afflicted, and all of them dumb."

The relative infrequency of cretinism at Mendoza much later (1877) is attested to by Lemos, who lived there and stated that he saw but few cretins (28).

More frequent cretinism was noted, at an earlier date, by French in the neighbouring department of La Rioja. In 1839 he wrote:

"Goitre, to a frightful extent, occurs in the province of La Rioja; few women are totally exempt. It is also frequently attended with mental imbecility, or complete idiocy. I have seen the tumor vie in size with the abdomen. It exists in this aggravated form in the valley of Famatima"(17).

Some twenty years later, Martin de Moussy (32) testified that cretinism was not as frequent in La Rioja or in Jujuy as it had been twenty years earlier.

The decrease in the prevalence of cretinism was accompanied, or preceded, by diminution in the number of deaf-mutes and of people with goiters (Table 4). While this table shows only the changes from 1869 to 1895 and 1914, the decrease in the prevalence of goiter in two places had been noted long before 1895.

In 1805, a writer known only by his initials, G.H.A. y P., (19) asked "Porque pues en esta ciudad (Salta, I. G.) se minoran de dia en dia estos fastidiosos tumores? Por la razon contraria, porque en Jujuy hay en todo tiempo los mismos?" (Why do these disgusting tumors diminish from day to day in Salta? Why do these remain the same in Jujuy?.) The water in Salta had remained the same.

Lemos (28), in 1877, writing of Mendoza, referred to "la disminucion progresiva del gran numero de bocios de tres decades a esta parte" (the progressive diminution here in the large number of goiters in the last three decades).

Perinetti and associates (36) referred to this paper and called it "interesting and well documented" but neglected the statement quoted in the preceding paragraph as well as all the other evidences of the marked decrease in the prevalence of goiter, cretinism and deaf-mutism throughout the Argentine Republic.

Among the more notable of these is the following statement in the introduction in the third census of the Argentine Republic (39, vol. 4, p. 269).

"En otro tiempo, principalmente en 1869, cuando se practico el primer censo, el número de habitantes atacados de esta afeccion era muy crecido; pero, posteriormente, debida, sin duda, al notable mejoramiento de la condiciones sanitarias de todo el pais, aquel número disminuyo enormemente, a tal punto que en la compilacion dal presente censo aparecío tan reducido que no dio lugara un agrupamiento especial." (Formerly, especially in 1869 when the first census was taken, the number of inhabitants attacked by this disease (goiter, I.G.) was very great; but later, due no doubt to the marked improvement in the sanitary conditions throughout the country, this number diminished tremendously, to such a degree that in the compilation of the present census it was not given a special designation.)

In this section, the history and character of goiter in the Argentine Republic have been reviewed. In spite of the great prevalence of goiter in certain large areas, and an enormous number of deaf-mutes, the incidence of cretinism was not great.

There was a great diminution in the number of goiters between 1869 and 1895 and from 1895 to 1914. In Salta this was evident as early as 1805 and in Mendoza by 1877.

Summary

The evidence is quite clear that goiter was not present in the Inca empire. Its first recorded appearance, and that is questionable, was about seventy years after the Conquest, in what is now Bolivia. It next appeared in Peru and, not much later, in the Argentine Republic. It was not reported from Chile until the nineteenth century. In the Argentine Republic, its incidence has been declining for a century, or more. In Peru, it seems to be spreading and in Chile, it seems to have been stationary from 1944 to 1949, and may now be declining.

Attention is called to the relatively small number of cretins and to the very large number of deaf mutes. The relation between goiter and deaf-mutism will be discussed in another paper.

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[b] New York University College of Medicine. Accepted for publication July 24, 1957.

[c] Lastres was guilty of a grave error of his own in writing: "Greenwald ha abordado este interesante problema de paleo-patologica, precisando su antiguedad en America. Nueva Zelandia e Inglaterra." (Greenwald has studied this interesting problem of paleopathology, establishing its antiquity in America, in New Zealand and England.) I claimed precisely the opposite, that goiter was not endemic either in the Americas or in New Zealand before the coming of Europeans and not in England until the eighteenth century (22).

INDEPENDENCE OF THE SURVIVAL TIME OF RAT ERYTHROCYTES AND THYROXINE INDUCED HYPERMETABOLISM[a]

C. E. HALL, J. B. NASH, AND O. HALL

Chromium-51, employed in the form of $Na_2Cr^{51}O_4$, has been repeatedly demonstrated to be a satisfactory agent for tagging erythrocytes in order to establish their mass and survival rate (5, 7, 10, 12, 14). Fortunately, the chromate ion in its hexavalent form binds firmly to the hemoglobin of the red cell, and is not, when released from damaged erythrocytes, incorporated into newly formed red cells. Furthermore, it appears that *in vitro* labeling of red cells with Cr^{51} does not alter their normal life span unless the concentration of the ion exceeds 10 μg per ml. of blood (5).

Recently we (8) and others (1) have shown that if donor rat blood is thus labeled, and injected into recipient animals, the subsequent decline in the radioactivity of the blood is such as to indicate a red cell half-life of approximately eight days. This figure is far less than that indicated by studies which have utilized other tracer substances (2–4, 6, 17), but these have had the disadvantages of having been administered via routes which rendered uncertain the rate of their entry into the red cell, and which, when liberated from disintegrating cells, are known to be incorporated into newly formed red cells.

Recently it has been contended that the red-cell survival is directly related to the metabolic rate (15). This inference was drawn from a comparison of the reported erythrocyte life-span for various animal species with the basal heat production reported for these same species by other investigators. Such a postulate suffers from the disadvantage, recognized by its proponents, that the information as to red-cell life-span and basal heat production was assembled from laboratories which had employed widely divergent methods of assessing the variables, and that the metabolic rates and red-cell survivals for any given animal species were not obtained with the same series of animals. Furthermore, the apparent correlation between basal heat production and red-

cell survival which is suggested by comparison of different animal species, is subject to the criticism that species differ from each other in many respects, and although basal metabolism happens to be one of these, its apparent relationship to red-cell survival may be purely fortuitous. Insofar as the data given on homeothermic mammals is concerned, it might be postulated equally validly that there is a direct correlation between body size and red-cell survival, a reasonable conclusion in view of the known fact that in such mammals "from mice to cattle, metabolic rate and body size are correlated" (11).

In this study a comparison was made between the survival of transfused erythrocytes in normal rats, and those concomitantly subjected to a dosage of l-thyroxine sufficient to augment the metabolic rate greatly, in order to ascertain whether any relationship such as that postulated could be found.

Materials and Methods

The recipient rats, weighing 125–145 gm., were females from our colony, the forebears of which were of the Holtzman strain. The donors were adult females of the same strain. Throughout the experiment the animals were maintained in aid-conditioned quarters and given Purina Laboratory Chow and water *ad lib.*

Red cells were obtained from approximately 22 ml. of blood which was withdrawn from the inferior venae cavae of three anesthetized donor animals into a syringe containing 5 ml. of Owen's ACD solution. To this was added 0.8 mc. of a $Na_2Cr^{51}O_4$ solution having a specific activity of 21.5 mc./mg. and the mixture incubated at room temperature for 30 mins. with occasional shaking. Following centrifugation and discarding of the supernatant, the packed cells were washed twice with equal volumes of 0.9 per cent NaCl solution and again centrifuged and the supernatants discarded.

Group 1, consisting of eight rats, was injected by tail vein, while under light ether anesthesia, with 0.35 ml. (141,900 cpm.) of packed tagged erythrocytes. Group 2, six rats, received 0.70 ml. (243,800 cpm.) in the same manner. Group 3, eight rats, were treated as group 1, but received, in addition, 1 mg./kg./day l-thyroxine. The l-thyroxine solution was made by suspending 50 mg. l-thyroxine in 49 ml. of physiological saline, adding, dropwise, 1N NaOH until the material dissolved, and then adjusting the final volume to 50 ml. Daily injections of 0.1 ml. of this solution per 100 grams body weight were subcutaneously administered to the rats in Group 3 throughout the course of the experiment, beginning twenty-four hours prior to blood transfusion.

One hour after the rats had received transfusion of cells blood samples of approximately 0.25 ml. were withdrawn from each by cardiac puncture and immediately weighed in planchets to the nearest 0.1 mg. These samples were then assayed in a scintillation counter in such a manner as to yield a count-

ing error of <2 per cent. Blood weights were converted to volumes by using Sherrington's estimate of 1.056 as the specific gravity of rat blood (16), and the number of counts/ml./min. ascertained. This was repeated at 7-day intervals thereafter for the ensuing three weeks excepting that the blood was, on these occasions, obtained from a severed tail tip, and the counts corrected to zero time.

The effect of hormone treatment on the metabolic rate was assessed by comparing the oxygen consumption of the animals in groups 1 and 3 at 7 and 14 days following transfusion. Oxygen consumption was measured at a constant temperature of 28° C. in a closed circuit respirometer capable of holding 8 rats, as described by Holtkamp *et al.* (9), and modified by Panos, Finerty and Wall (13). Pure O_2 was delivered into the apparatus and the expired CO_2 was absorbed on soda-lime. For each run, half controls and half thyroxine-treated rats were studied concomitantly and the O_2 consumed was calculated from the average of three consecutive 15-minute trial periods, which were begun after allowing each animal at least 15 minutes to become accustomed to confinement.

Results

The rate of disappearance of radioactivity from the peripheral blood was identical in each of the three groups. Oxygen consumption of animals in groups 1 and 3 indicated that during the period of greatest rate of decline in blood radioactivity, *i.e.*, in the first two weeks, the metabolic rate of rats treated with l-thyroxine was approximately 80 per cent greater than that of controls. Although the oxygen consumption was not measured on the 21st day, it seems reasonable that the metabolic rate would have remained elevated since hormone administration was continued at the same dosage throughout the experiment.

The half-life of transfused red cells averaged five days. Although there was great uniformity of response amongst the animals of group 1, a greater range of response was encountered amongst those of groups 2 and 3. The data are summarized in table 1.

Discussion

Statistical analysis of the data indicated that whereas in the dosage employed l-thyroxine elevated the metabolic rate by about 80 per cent, the survival of transfused red cells was not affected thereby. It would thus appear that red-cell survival is not correlated with metabolic rate in the rat, at least insofar as normal and elevated metabolic rates are concerned.

Data obtained in our laboratories (8) and elsewhere (1) had

TABLE 1

Survival of Cr^{51} Labeled Erythrocytes and Oxygen Consumption of Control and l-Thyroxine Treated Rats.

	Data	Group 1	Group 2	Group 3
	No. of Rats	8	6	8
	Body Wt. Gm.	130 ± 4*	130 ± 5	129 ± 4
Day	Per Cent Original Activity in Blood	30 ± 1.6	31 ± 5.1	40 ± 8.6
7	O_2 Consumed Liters/kg./hr.	1.79 ± 0.29	...	3.22 ± 0.18
Day	Per Cent Original Activity Remaining	10 ± 1.5	11 ± 2.4	14 ± 3.0
14	O_2 Consumed Liters/kg./hr.	1.85 ± 0.05	...	3.32 ± 0.18
Day	Per Cent Original Activity Remaining	2.6 ± 0.4	2.2 ± 1.1	4.8 ± 1.9
21	O_2 Consumed Liters/kg./hr.	...	...	...

* ± S.E. of Mean.

indicated that the half-life of the rat erythrocytes as determined by Cr^{51} labeling was about 8 days. The present studies indicated a half-life of only about 5 days, although the maximum life-span was substantially the same as had been estimated from the other studies. There are three respects in which the procedures employed in the present study differed from those adopted previously, which may explain this discrepancy. In the first place the specific activity of the radioactive material used in this study was much greater than that used heretofore, as a result of which the tagged cells received less chromate ion, but greater radioactivity. Secondly, whereas in the previous study the cells had been diluted with an equal volume of isotonic saline prior to transfusion, packed red cells were administered on this occasion. Thirdly, a larger total number of cells was transfused into each animal in the present series.

It seems unlikely that the latter could account for the difference in response. For although all of the animals in the present experiment received a larger number of cells than did those of the earlier experiments, the animals of group 2 received twice the volume of packed cells as those in groups 1 and 3, yet the erythrocyte helf-life was identical in each of these groups.

There remains the possibility that the transfused erythrocytes sustained traumatic injury resulting in premature destruction, either because of the higher amount of radioactivity to which

they were exposed or because of mechanical injury as a consequence of injecting packed cells intravenously through a 23 gauge needle. Further studies designed to elucidate this and other problems relative to factors which affect the survival of transfused red cells in the rat are in progress.

Summary

Erythrocytes from donor rats were labeled with $Na_2Cr^{51}O_4$, and injected into control and l-thyroxine treated female rats. Animals comprising one group of controls each received approximately 0.35 ml. of packed labeled cells, those of the other control group received 0.70 ml., and a group of animals, at the lower dosage, each received 1 mg./kg./day of l-thyroxine subcutaneously beginning 24 hours prior to transfusion. The rate of disappearance of radioactivity from the circulating blood was identical for each of the groups, although thyroxine treated rats were found to have an approximately 80 per cent increase in O_2 uptake. It is concluded that thyroid induced hypermetabolism does not affect the life-span of transfused erythrocytes.

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Q FEVER IN TEXAS[a]

J. V. IRONS,[b] R. B. EADS,[c] AND J. E. PEAVY[d]

Since the discovery of Q fever in Australia (6), it has been shown that this disease is world-wide in distribution (2, 7, 10). The importance of airborne infection (1, 5, 15), in vicinity of infected livestock has been well established. Previous reports of Q fever in Texas concerned an explosive outbreak (10, 15) in slaughterhouse and stockyard workers at Amarillo, serological evidence (14) of past infections in slaughterhouse workers at Fort Worth, and scattered cases (11, 13) in South Texas. A survey of arthropods (9) in a selected but limited area of Southwest Texas was negative for *Coxiella burneti*, although earlier reports described the finding of the agent in Texas ticks (4, 12).

The present report concerns data from September, 1948, through December, 1955, on 64 laboratory confirmed Q fever human cases and the infection in 8 dairy herds. Other pertinent data relative to Q fever or *Coxiella burneti* are given. The methods of investigation already have been described (8, 11). Diagnosis of Q fever was based on clinical, epidemiological and laboratory findings. The laboratory aids were most important and included the complement fixation test or identification of *Coxiella burneti*, the causative agent, on specimens from man or animals.

Clinical, Epidemiological and Laboratory Findings on 64 Cases of Q Fever

From September, 1948, through December, 1955, 64 cases of Q fever were recognized. The clinical data were fragmentary except on the 21 hospitalized patients, which were similar to those described by others (3, 7, 13). The onset tended to be acute, with fever, chilly sensations, headache and other body aches or pains. Chest discomfort and nonproductive cough were frequent complaints. X-ray chest plates of those more severely ill commonly revealed pneumonitis or atypical pneumonia. Physical examination usually revealed little of note. A rash was not observed. There was considerable variation in severity of the illnesses; some illnesses were mild and were described as influenza-like

attacks. Fever frequently reached 103–104° F. and the febrile period frequently persisted 4–7 days or longer. Physicians frequently stated that the febrile period apparently was shortened by use of the broad spectrum antibiotics. Relapses were uncommon. Convalescence often was prolonged, particularly with those severely ill or the older patients. One patient contracted tuberculosis shortly after recovery from Q fever. There were no fatalities.

The ages of the 64 cases varied from 17 to 75 years. Forty-eight of the 64 cases were males. Cases occurred during all seasons of the year, but predominantly in the third quarter (Table 1). The great majority of cases were farmers, ranchers, dairymen or their wives, butchers, veterinarians, or others associated with livestock, particularly cattle. The remainder of the cases represented almost as many occupations.

TABLE 1

Seasonal Occurrence of Q Fever in Texas, 1948–1955

Month of Onset of Illness	No. of Laboratory Confirmed Cases	Month of Onset of Illness	No. of Laboratory Confirmed Cases
January	6	July	3
February	5	August	8
March	4	September	10
April	6	October	3
May	6	November	4
June	4	December	5

The possibility of infection acquired from drinking raw milk could be ruled out in a few cases who stoutly denied drinking raw milk or eating raw milk products. Inhalation of dust around dairy barns, cattle trucks and livestock pens was a possible mode of transmission in the majority of cases. Cattle loading pens frequently are located near highways and many more people come near cattle and cattle trucks at auctions, fairs, rodeos, etc. Infection from association with sheep or goats appeared to be ruled out with the possible exception of 3 cases. Tick bites seemed not to be an important source of infection, since few could recall a recent tick bite prior to onset of illness. With the exception of four cases at Fort Worth and one at San Antonio, those ill were not residents

of the larger cities; the great majority resided south of Austin, in the southern part of the state, as shown in figure I. Forty-six of the cases resided as far south as San Antonio. Several of these cases resided at or near Kingsville in Kleberg county; an infected dairy also was near Kingsville. Those ill resided in 29 counties, but 35 of the cases resided in Duval, Kleberg, Bee, Uvalde, Cameron, Nueces, and Zavala counties in south Texas. Duval and Kleberg counties accounted for 18 of these 35 cases. It seemed probable that these 64 cases were only a small fraction of the actual number of cases.

The clinical laboratory findings were not noteworthy. The few leucocyte counts done usually were in the normal range. The few blood cultures made were negative. Agglutination tests for typhoid and tularemia were negative, but seven cases showed Brucella agglutinins; two of these patients were veterinarians. It should be noted that unlike most rickettsial diseases, in Q fever the Proteus OX agglutinins are not increased.

By the end of the third week after onset of illness complement fixation titers of 1:40 or higher, such as 1:320 and 1:640 were frequently found. A relatively high titer was found to persist for several weeks after the illness. A four-fold significant rise in titer usually was demonstrable provided the first bleeding was done before the 10th day and the second not earlier than the 17th day after onset of illness. Provided the early blood specimen was not taken too late, the collection of the second specimen could be delayed several weeks. A high titer on a single delayed bleeding was considered confirmatory when other findings were characteristic. Numerous suspect cases were unconfirmed, apparently because of inadequate investigation and failure to obtain requisite blood specimens.

The agent was recovered from the blood of one case and was quite typical of *Coxiella burneti*. Infection with this agent resulted in an enlarged spleen in mice and guinea pigs, in which characteristic small rod-like organisms were demonstrable in Macchiavello-stained preparations. Infected mice, hamsters and guinea pigs showed complement fixing antibodies in high titers, both with antigens prepared from American (Nine Mile) and Italian (Henzerling) strains of *Coxiella burneti*.

Studies on Q Fever with Reference to Cattle, Goats, and Sheep

In addition to the 3 dairies previously reported (9, 11), *Coxiella burneti* was demonstrated in 5 other dairy herds. All 8 dairies were located in south Texas, well south of San Antonio, as shown in figure I. Four of these dairies were singled out in the

FIG. I. Q Fever in Texas. September 1948 through December 1955.
D-1—Q fever dairies (8 in 6 counties).
1—Number of laboratory confirmed cases of Q fever (64 in 29 counties).

investigation of 4 human cases of Q fever. Five additional cases were found among 29 employees or owners of the 8 dairies. Six others showed titers from 1:10 to 1:80. One to three animals were found to be "shedders" in these herds, which were relatively small. Eighty-nine dairies in south Texas failed to show the presence of "shedders"; however less than half of these herds were tested repeatedly. Because of a considerable dilution from pooling milk (11) many tests probably were relatively insensitive; *C. burneti* frequently was limited to one quarter of the udder and demonstration of the ID_{50} of milk from a "shedder" rarely exceeded 10^{-3}. In 1951, "shedders" were not found in 2 dairies in which the agent was demonstrated in 1948. No new "shedders" were encountered during a three-year observation period on 2 other dairies.

The agent was not found in the blood or nasal swabbings of "shedders" or their calves. Unfortunately, it proved difficult to obtain placental tissues and this part of the study was incomplete. "Shedders" and their calves tended to show significant serum complement fixing titers.

Five per cent of 121 sera from dairy cattle and 2 per cent of 97 sera from beef cattle in south Texas showed complement fixation titers at 1:10 or greater.

Only one of the 8 dairies sold raw milk to consumers. The presence of a "shedder" in this small herd was found after a customer on the milk route became ill. It was difficult to rule out the possibility of infection from drinking raw milk in many cases. The possibility of infection from drinking pasteurized milk seemed quite remote. Numerous tests of pasteurized milk invariably were negative for *Coxiella burneti*.

None of the tests of more than 100 dairies in central Texas showed the presence of "shedders." The great majority of these dairies were tested more than once and many were subjected to repeated tests over a 3-year period. Seventy sera from abattoir workers and dairymen in central Texas were essentially negative. Fifty-nine sera from dairy and 89 sera from beef cattle in central Texas failed to show serological evidence of Q fever.

None of the tests of milk from 27 dairies in north or east Texas showed presence of a "shedder." None of the sera from 17 employees of these dairies showed serological evidence of past infection.

A few random pools of goat and sheep milk collected in southwest Texas were negative. Five per cent of 261 goat sera from southwest Texas showed titers at 1:10 or greater. Some of these specimens were tested in investigating two cases of Q fever.

Results of Serologic Tests in Domestic and Native Mammals in Endemic Areas

Several sera from cats, dogs, domestic rats and hogs were negative. The occasional finding of low or moderately high titers in sera from the pack rat, *Neotoma micropus*, and the cotton rat, *Sigmodon hispidus*, were of questionable significance. It was noted that pack rats showing a titer usually were trapped near water holes frequented by cattle, but attempted recovery of *Coxiella burneti* from the tissues of animals showing titers failed. Experimentally it was found that a significant rise in complement fixing titer followed the inoculation of *Neotoma micropus*. *Coxiella burneti* was not recovered from the spleen, brain or kidney of these animals several weeks later.

Failure to Recover Coxiella burneti from Pools of Ticks and Other Arthropods

All tests of field-collected ticks and other arthropods were negative. The arthropods included 15 species of ticks, 5 species of mites, 7 species of lice and 9 species of fleas. In obtaining ectoparasites, over 4,000 animals representing 30 species (principally rodents) were examined. Many pools of the Lone Star tick, *Amblyomma americanum*, and the spinose ear tick, *Otobius megnini* were taken from several counties. Many pools of *Amblyomma americanum* were taken at Camp Bullis near San Antonio. Several pools of *Ornithodoros turicata* and *Ornithodoros talaje* were included.

In addition to large numbers of ectoparasites, pools of mosquitoes, house flies, stable flies, horse flies, blow flies, flesh flies, *Triatoma* bugs and dung beetles taken around dairy barns in endemic areas were tested with negative results.

Coxiella burneti was found in small pools of the ticks, *Ornithodoros turicata* and *Ornithodoros talaje* following feeding experiments on laboratory inoculated animals. These findings seemed to indicate that the test procedures were satisfactory.

Summary and Discussion

The principal clinical, epidemiological and laboratory findings on 64 cases of Q fever were presented. These cases occurred in the 7¼-year period from September, 1948, through December, 1955. There was evidence that many additional cases occurred but were not recognized. Clinical features in this series of cases frequently showed similarity to those of other acute febrile illnesses. The rash which is a feature of some rickettsial diseases is not characteristic of Q fever.

The Proteus OX agglutination test was negative. A diagnosis of Q fever essentially is a laboratory diagnosis for which specific serological tests are most useful. The complement fixation test was used primarily, both as a diagnostic and epidemiological aid in this study.

The great majority of cases were in adult males who were residents of farms or small urban communities in south Texas. Cattle seemed to be the most probable source of infection. Sheep and goats seem to be perhaps of less importance as a source of infection than an earlier study (11) had suggested.

Surveys of the infection in livestock suggested that enzootic areas were more prevalent in the southern part of the state. The 8 dairies in which the infection was investigated were located in south Texas. Similarly, in southern California (1) cattle were shown to be of much more importance than sheep or goats as sources of human infection. Failure to find the agent in many pools of ticks cast doubt on their importance in this disease.

The finding of endemic and enzootic Q fever primarily in south Texas and the concentration of cases in the more southern counties (11), particularly Kleberg and adjoining counties (13), points up the need for further investigation.

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[b] Director of Laboratories, [c] Senior Entomologist and [d] Director of Communicable Disease Control, State Health Department, Austin, Texas, Accepted for publication August 30, 1957.

THE PRESENT SITUATION IN REGARD TO THE SEROLOGIC TESTS FOR SYPHILIS

KINCH C. KNOLLE[a]

Laboratory Tests for Syphilis

Many tests have been devised since Wasserman's first report (11) of applying the complement-fixation reaction to syphilis in 1906. Each of these tests has certain advantages, but none has proved better than the others in all respects. The tests have a high degree of accuracy when compared to other clinical laboratory procedures, but the conflicting results sometimes encountered have added confusion to the often difficult problem of the diagnosis of a disease that at times presents a great variety of symptoms from the mildest to the most severe, and at other times has no symptoms at all.

Typical of the inconsistency of the disease, there are present not one but two separate and distinct antibodies or groups of antibodies. This unusual situation makes it possible to have two groups of laboratory tests. One is based on reactions with a nonspecific, non-treponemal antibody and the other is based on reaction with the true treponemal antibody.

Non-treponemal Tests for Syphilis

A non-treponemal antibody, sometimes called "reagin," is quite consistently found in the blood stream of persons infected with syphilis. This antibody, however, is not reactive with the living *Treponema pallidum*, the causal organism of the disease. Its presence is demonstrated by reactions with a nonspecific lipoidal antigen obtained by alcoholic extraction of powdered beef-heart tissue.

The presence of this non-treponemal antibody has not been adequately explained. It probably is an antibody that is produced by the body as a response to antigenic stimulation not by the spirochete itself, but by some substance which is coincidentally present whether it be a degradation product of the spirochetes, a degradation product of the patient's own tissue on destruction

by the spirochetes, or possibly a combination of the spirochete with a product of tissue destruction.

Laboratory tests based on the reaction between this non-treponemal antibody and the non-specific lipoidal antigen that have been widely used are the Kolmer complement-fixation, Kahn standard, Kahn presumptive, Kline diagnostic, Kline exclusion, Eagle flocculation, Eagle complement-fixation, Hinton flocculation and Mazzini microflocculation tests (5).

An attempt was made to obtain the actual reactive components of this lipoidal antigen and thereby improve the tests (8). This purified antigen is referred to as "cardiolipin-lecithin" antigen. Its advantages over the older lipoidal antigen are reflected in the tests by greater sensitivity, by obtaining fewer "false positive" results, and by more consistent results obtained because of the possibility of a more accurate standardization.

Cardiolipin-lecithin antigen has been substituted for the lipoidal antigen in the Kolmer, Hinton, and Mazzini tests (10). This has resulted in a change in the reactive properties of these tests without giving any indication of this change by altering the names of the tests. The Kline standard (10) employing cardiolipin-lecithin antigen has replaced the Kline Diagnostic and Kline exclusion tests. The Eagle tests are unchanged but are seldom used. The Kahn standard test is the same as it was when originally published in the 1920's (3). A Kahn procedure using cardiolipin-lecithin antigen has been described, but it has not gained wide usage (10).

Several new test procedures employing cardiolipin-lecithin antigen have been published. The VDRL (Venereal Disease Research Laboratory) slide test (10) is gaining tremendous popularity. The VDRL tube test, the Rein-Bossack, and the APHA (American Public Health Association) reference tests (10) are available.

Significance of Non-treponemal Tests for Syphilis

It must be emphasized that results of laboratory tests (in actuality, determinations of the absence or presence of antibody and, if extended, determinations of the quantity or titer of the antibody present), can be as conflicting and confusing as the symptomatology of syphilis. The following discussion will be an over-

simplification in an attempt to present it in a logical manner. Many exceptions to the statements will be encountered.

In the first few weeks after infection the tests are nonreactive and remain so until the reagin or non-treponemal antibody production has occurred. The tests do not become reactive until approximately one month after infection. When the non-treponemal antibody first makes its appearance in the blood stream, due to the different characteristics of the various tests, conflicting results are usually encountered. As the non-treponemal antibody increases, all tests become reactive and quantitative procedures are necessary to determine the titer. The titer rises rapidly and remains high throughout the rest of the primary stage and extending into the secondary stage of the disease. There is considerable variation in the maximum titer in different individuals. If successful treatment is given in the early stages of the disease, the non-treponemal antibody quickly disappears from the blood stream. Non-reactive results are expected in from three to six months.

No prediction can be made as to the expected results of laboratory tests in cases extending into the latent stage. The antibody titer is a reflection of antigenic stimulation and is influenced by the patient's ability to produce antibodies and the amount of antigenic stimulation present. Titers range from very high to so low that conflicting results are obtained in the qualitative procedures.

One of the outstanding challenges for the physician to encounter is a case showing only a low titer or even conflicting results. He must determine from all evidence available whether or not this is a case of syphilis. The patient may have received treatment and no longer remembers or admits the fact, or he may have received sufficient penicillin or other antibiotics in treatment for other diseases or infections to cause this low titer. The natural defense mechanism of the body may have reduced the infection to a point resulting in insufficient antigenic stimulation for antibody production. The physician cannot overlook the fact that the reaction may be due to some condition other than syphilis and is, therefore, in the "false positive" category.

In the later stages of syphilis the vast array of symptomatology makes its appearance usually with the accompaniment of definite serologic results.

The quantitative test procedures, reported in terms of titers, are most valuable as a post-treatment guide. The results, though erratic, usually revert to non-reactivity following successful treatment. As a generalization, the longer a patient has had syphilis, the longer it will take for a disappearance of antibodies. This may be a matter of months after treatment of early syphilis or a period of years after treatment of late syphilis. The consistent reduction of titer is the important fact; the time it takes a relatively unimportant. A sustained rise in titer at any time after treatment tends to indicate a relapse or a new infection.

The examination of the spinal fluid is often indicated for proper diagnosis and always before a patient is dismissed as having been cured. Non-treponemal antibodies found in the spinal fluid are produced by the nerve tissue and only after antigenic stimulation. Therefore, antibody present in the spinal fluid is indicative of central nervous system involvement. A cell count and total protein determination may be invaluable in establishing disease activity.

The demonstration of the causal organism in exudate from the primary chancre, or aspiration of involved regional lymph nodes in primary and secondary syphilis, and from certain other lesions of secondary syphilis by darkfield microscopy is diagnostic of the disease.

"False Positive" Reactions

The term "false positive" as applied to reactive results in conditions other than syphilis is misleading. In most instances these are true reactions in the test caused by antibodies of diseases other than syphilis. Other treponemal diseases such as yaws, give reactions in tests for syphilis. Reactions in cases of leprosy are expected. Malaria, severe respiratory infections, recent immunizations, collagen diseases, and probably many others will at times cause false positive results. In fact, it may be stated that any disease or condition other than normal will tend to give false positive reactions in a ratio greater than is found in normal individuals.

Many of these false reactions are transient. Within a few months non-reactive results are often obtained. A persistent false positive may be indicative of an unsuspected chronic disease.

Dr. Kahn presents this differently (4). With his "universal" reaction he shows that his test can be made to give reactions in nearly any individual simply by manipulations causing increased reactivity. In cases of disease this latent reactivity is increased to a variable extent. The differences in the pattern of results obtained by these manipulations of the test in various diseases seem to be fairly consistent. It may be, therefore, the nontreponemal antibody is found in other diseases as well as in syphilis.

Treponemal Tests for Syphilis

The specific antigen, *Treponema pallidum*, is obtained from rabbits having been inoculated with virulent spirochetes. A multiplicity of test procedures are available to demonstrate the reaction between the specific antigen and the true antibody that is produced by the tissues as a response to the antigenic stimulation of the living spirochete.

The TPI (*Treponema pallidum* immobilization) (6) test uses living spirochetes as the antigen. The antibody in the serum of a syphilitic in combination with complement causes the spirochetes to lose their motility. The TPCF (*Treponema pallidum* complement-fixation) (9) uses an extract of the spirochete as the antigen in a complement-fixation reaction. The TPA (*Treponema pallidum* agglutination) (1) and the TPIA (*Treponema pallidum* immune adherence) (7) uses a suspension of killed and preserved spirochetes as the antigen. Only the TPI and TPCF tests have been developed into practical test procedures. In the experimental stage is the RPCF (Reiter Protein complement-fixation) (2). The antigen for the test is an extract of the Reiter strain of *Treponema pallidum*.

Significance of Treponemal Tests

Sufficient time has not elapsed to properly evaluate the accuracy of the comparatively new tests using *Treponema pallidum* as the antigen and demonstrating the presence of a true treponemal antibody found in cases of syphilis. A review of the literature published on this subject indicates that these tests are more specific (give fewer false reactions).

Other treponemal diseases as yaws, etc. will give reactions with the specific antigen tests. The time required for the appear-

ance of reactive results in the TPCF test approximately parallels that of the non-treponemal antibody tests. The TPI test will not be reactive until sometime later. It may be a few days to a few weeks before it shows a reaction. Cases of early syphilis treated successfully will show a reversion to non-reactive results, but cases untreated for some years fail to revert and will probably remain reactive for the lifetime of the patient regardless of his clinical status. Little is known about the significance of antibody titers. The presence of treponemal antibody is being used to indicate syphilis; the absence is supposedly indicative of not having syphilis. Because of the greater difficulty encountered in the procedure of the TPI test, the TPCF test is more generally used, especially as a preliminary test.

An evaluation study is being conducted by the United States Public Health Service under direction of the Serology Evaluation and Research Assembly. The study is expected to be completed and reported during the next year. This study should establish the relative merits of the various tests.

Practical Considerations

Demonstration of *Treponema pallidum* by darkfield microscopy is diagnostic of syphilis. It should be used not only on suspected primary chancres, but also on swollen lymph nodes and possible condylomata lata whenever present and unexplained.

The multitude of available serologic tests for syphilis is resolved into two groups, non-treponemal and treponemal.

At the present time in diagnosis of syphilis, the results of one (or more, if preferred) non-treponemal tests, including quantitative results if qualitative tests are reactive, should be obtained. In cases giving definite reactions, having typical symptomatology and appropriate history, no further tests are mandatory. In cases showing no or inconclusive symptoms and indefinite clinical history, especially if the test or tests are of low titer or are in disagreement, it would be advisable to obtain results of treponemal tests. The TPCF is the most readily performed (the TPI may be used to supplement the results of the TPCF). Reactions in the treponemal tests are strongly indicative of the presence of syphilis. It must be recognized, however, that the degree of accuracy of these tests has not been absolutely estab-

blished. An interpretation must be made with caution until more statistical evidence of the accuracy of the test is available.

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* Serologist, Division of Laboratories, Texas State Health Department.

AN ELEMENTARY DISCUSSION OF THE DESIGN OF A PUNCH CARD SYSTEM EXEMPLIFIED BY ONE FOR NEUROPHYSIOLOGY

SIDNEY OCHS[a]

Introduction

With the usual card filing system, information becomes rapidly more difficult to find as any sizable number of cards is accumulated. Cross-filing necessitates making out an additional one or more cards to file under the appropriate subject heading and is time consuming—as is the need to sort cards for proper return to the file.

The simple hand type of punch card recently coming into wider use is well suited to one's private needs. These punch cards have space on front and back for the information material and contain holes along the edges which are numbered and can stand for the various subjects or categories. A given card contains information on one or more of the categories of the system and the appropriate hole corresponding to the category is cleared out to the edge with a punch. When from a stack of cards those containing that desired category are to be selected, a needle is passed through that hole number; all cards so punched will fall down and be available for reference. Of cards selected for one category, a needle may again be passed through another hole to select for a second category and so on. The group selected becomes smaller and more specific in its area of information content. It can be seen that a large number of specific topics can be selected, for if there are 50 holes, each hole may for example be theoretically paired with each of the others.

The problem of devising an adequate breakdown of a subject into exclusive and relatively equal categories is particularly difficult for a mixed field of interest such as neurophysiology represents. Parcellation into a large number of specific subjects would require too many holes and would be unwieldy in use. The solution is to pick those categories with the required coverage. These should be of an intermediate nature, between some-

thing as general as say "drugs" or as specific as "epinephrine"; something which "autonomic drug" would represent. As our interest is in neurophysiology, we would not want to give over a hole to "autonomic drugs." We can however, fulfill our needs with the general term "drugs" in combination with "autonomic N.S." We then also have the term "autonomic N.S." for use in other contexts. Thus, appropriate general terms outside of the proper domain of neurophysiology, combined with the intermediate terms within it, makes for a flexible wide coverage of related fields.

Some subjects may clearly not be in the categorized field—such as those representing a completely different interest. In this case it would be better to keep such cards separate, and if necessary devise a separate punch card system for them.

In this paper I will present the system devised for my use to serve as a model for the construction of a system by those in other heterogeneous fields of interest.

The "family" grouping

Each "family" group heading—"object," "variable," "function," takes an edge of the card. It is convenient to separate out and keep these families together. The separation into families makes the system easier to use for it is in accord with the logic of our language usage, where we think of a thing or an object acted upon by a variable, or a function of an object or thing changed by or producing a variable. Some attempt may be made to arrange each family in some order, *i.e.*, of size or complexity. The fourth family, "classificatory," takes the fourth side of the card. It helps to select for special kinds of information—*i.e.* whether the card is concerned mainly with *e.g.*, technique or theory.

Examples

Perhaps the best way of showing how the system is used is to describe how some typical cards containing paper abstracts are punched.

The first example is, "Spreading cortical convulsions and depression," van Harreveld and Stamm, *J. Neurophysiol.* 1953, 16: 352. The categories punched are "cortex," "depression," and "convulsions."

This card will come down with the "cortical" group in case one wishes to review cortical physiology papers. In like fashion it will come down with the "convulsion" group or the "depression" group.

The title of the next example is, "The influence of stretch on the membrane potential of the striated muscle fiber." Ling and Gerard, *J. Cell. Comp. Physiol.*, 1949, 34: 3. The categories punched are "membrane," "muscle," and "electrical properties." Membrane studies of muscle are relevant to those of nerve and this paper would be included with the nerve group when "membrane" is selected. It is a moot question whether to punch the "mechanical" category or not in this case. It seems best not to and to keep it either for papers concerned directly with the mechan-

TABLE 1

The Punch Card System

Object	*Function*
4. Fluids and secretions	48. Anatomy (distribution)
5. Membrane	49. Electrical properties
6. Cell and cell particulates	51. Slow changing potentials
7. Nerve	53. Spikes and broad discharges
9. Muscle	54. E.E.G.
10. Myoneural junction	55. Excitability and conduction
11. Ganglion	57. Permeability
14. Tracts and connections	58. Organization and pattern
17. Spinal cord	60. Emotion and stress
18. Sub-cortical	61. Behavior, conditioning
19. Reticular formation	62. Reflexes
21. Cortex	63. Posture, locomotion, 'movements'
24. Sense organs and system	64. Sensation
25. Brain or C.N.S.	65. Mentation (consciousness, sleep)
26. Autonomic N.S.	66. Depression (fatigue)
27. Organs and tissues	67. Narcosis
28. Lower species and plants	69. Circulation and fluid balance
Variable	70. Metabolism and energetics
31. Temperature	71. Respiration and asphyxia
32. Mechanical	72. Growth and development
33. Light	73. Biotic (reproduction, feeding)
34. Sound	74. Convulsion
37. Electrical	*Classificatory*
38. Biochemical (chemical)	76. Theoretical
39. Ions	77. Technique
43. Drugs	78. Review or bibliography
44. Hormones	
45. Lesion or pathology	

The numbers listed are the hole numbers of the cards. Notice that unused holes are scattered through the system to provide for future additions.

ical properties of muscle or in combinational fashion as with "sensation" to refer to touch.

The last example is, "A reinterpretation of the mathematical biophysics of the central nervous system in the light of neurophysiological findings," Rashevsky. *Bull. Math. Biophysics*, 1945, 7: 151.

The categories punched are "brain or C.N.S.," "organization and pattern," and "theoretical." "Brain or C.N.S.," is punched because of the nonspecificity of the location concerned; cortex, sub-cortex, or spinal cord. "Organization or pattern" is selected to cover the notion of the functional arrangement of connections in a region. The category "theoretical" is of course central with this paper and subjects which are mathematical, philosophical or speculative are grouped in that category. Some of the other categories such as "transmission," or "tracts and connections" could conceivably be also punched. But these have different connotations. "Tracts and connections" refers to the connector of one area with another, *e.g.*, the pyramidal tract. "Transmission" refers to the functional continuity of one region with another, not the form of transmission which is represented by "organization and pattern."

It is a good plan to keep the number of category holes used for each card small. If a conscious attempt to restrict the categories punched to two or three is not followed and a card is punched for minor points, then its inclusion with more desirable categories will increase the number finally sorted by sight. If done persistently this habit will result in a diffusion of the desired results.

The use of more specific categories was resisted because it tends not only to "pocket" cards away but because the number of such specific interests can grow too easily and defeat the purpose of constructing and using a simple system.

Comments on Some of the Categories

The use of more than one term of similar functional nature for one category is a "lumping" device to keep the number of categories small. In some cases a term was used as a symbolic reference to the whole area included. Some of them may need additional specification to make their use clear:

(27) *"Organs and tissues,"*—Included are papers which deal with a particular organ—lung, kidney, etc.

(28) "Lower species and plants"—lower species for animals refers to invertebrates and below. Single celled organisms are grouped with (6) cell and cell particulates.

(73) "*Biotic*"—represents papers concerned with reproduction. feeding, ecology, heredity, evolution, etc.

(72) "*Growth and development*"—contains ontogeny and also regeneration.

Final Comments on Starting the System

After devising a system with the best possible division into categories, a trial of some 100 cards should be undertaken. This will help considerably to indicate the necessary new modifications. After several changes a system should emerge which will be satisfactory. Even then, leaving half the number of holes available for future expansion is wise.

The desire to select uniquely for specific cards should be resisted for one of the advantages of the system is a degree of broadness in the cards which come down. The trouble in visual selection of some ten to twenty cards representing a final selection is minor compared to the flexibility of use and recovery of information afforded by such a punch card system.

Acknowledgment

The author wishes to express his indebtedness to the stimulating discussions at the Illinois Neuropsychiatric Institute in Chicago, concerning the construction of punch card system started by Professor R. W. Gerard.

[a] Department of Physiology. Medical Branch, The University of Texas, Galveston, Texas. Accepted August 16, 1957.

[b] The cheap and readily available one used is the Unisort Analysis Card, Y9, Hadley Co., which contains 91 holes numbered from 1 to 91 along the edges, 5 x 8 inches in size.

SPECTROPHOTOMETRIC EXAMINATION OF CIRRHOTIC LIVERS FOR 3-METHYLCHOLANTHRENE FROM WHITE PEKIN DUCKS TREATED WITH THIS CARCINOGEN[a]

R. H. Rigdon, Harold Eason, and Jack Neal

Carcinogens are known to produce necrosis, cirrhosis, and tumors in the liver of experimental animals (Strong, 1944) (Andervont, 1939) (Davidson, 1923) (Twort and Twort, 1932) (Von Glahn, *et al.*, 1938). A peculiar hepatic lesion was observed in some white Pekin ducks following the subcutaneous, intramuscular or dermal applications of methylcholanthrene (Rigdon, 1955). It was either a focal or diffuse lesion characterized by a marked proliferation of reticulum without an accompanying hyperplasia of either hepatic cells or bile ducts, such as one usually finds in mammalian livers injured by a variety of chemical agents. Ascites frequently was present in the ducks with this atypical cirrhosis. Since all the birds showing this hepatic lesion had received methylcholanthrene, an attempt was made to demonstrate this carcinogen in the liver by spectrophotometric techniques.

Lorenz and Shimkin, in 1941, injected mice intravenously with methylcholanthrene and extracted the lung for this carcinogen. They found that after four days the amount of methylcholanthrene had diminished to one-tenth of its original value. More recently, Hadler *et al.* (1957) studied the route of elimination of methylanthrene from the skin of mice and found that its disappearance is quite rapid and involves a transient protein bound phase.

Methods and Material

Young adult white Pekin ducks were used in these experiments. Methylcholanthrene[b] in a 0.25 percent concentration in acetone was applied to the skin by dropping it from a pipette. The amount applied each day varied from 0.5 to 1.5 ml. Methylcholanthrene when put into the trachea was suspended in mineral oil (100 ml. + 1.5 gms.); 0.5 ml. was put into the trachea through the external larynx with a small catheter. For oral administration methylcholanthrene crystals were put into gelatin capsules (50 mg. each). For

subcutaneous, intramuscular and intracerebral inoculation, methylcholanthrene crystals were compressed into pellets (2–5 mg. each) and injected through a small trochar into the respective sites. The total amount of methylcholanthrene given to each duck and the interval between the last injection of the carcinogen and death of the birds are given in the different experiments. Specimens of tissue from the skin, trachea and liver were extracted for methylcholanthrene. Some tissues were used fresh, while others were fixed in a 4 percent solution of formaldehyde and kept for varying intervals before extraction.

In the process of extraction the tissues were cut into pieces approximately 10 mm. square, dehydrated in an oven at 67° C., placed in a paper thimble and extracted in a sohxlet extractor for six hours. The benzene extracted solution was then filtered through Merck's aluminum oxide (chromatograph column, 38 mm. diameter x 230 mm. length) after which the column was washed with 250 to 500 ml. of benzene. The benzene was then evaporated and the residue was redissolved in 500 ml. of petroleum ether and filtered through the same size column of aluminum oxide as used above. Methylcholanthrene appeared as a distinct purplish-blue florescent band on the filter when viewed in an ultraviolet light (3650–3663A°). The area on the filter showing the "blue-band" was removed by forcing the column of aluminum oxide out of the chromatographic tube. The "blue band" was separated from the other bands with a sharp knife while viewing it under the ultraviolet light. This material was then put on a filter paper in a funnel and depolarized with 10 ml. of methanol. The resulting filtrate was clear. The ultraviolet absorption curve of this methanol solution was obtained in a Beckman spectrophotometer and compared with that from methylcholanthrene crystals dissolved in methanol. The absorption curve of the latter is shown in figure I.

Experimental

The liver from 16 ducks was studied for the presence of methylcholanthrene. Five showed the cirrhosis previously described (Rigdon, 1955), while 11 of the livers were microscopically normal. Table 1 gives the results of this study.

Figure II shows the absorption curves as obtained from the liver of 7 different ducks. Only one of these, D-1027, shows a curve that could be considered as consistent with that resulting from methylcholanthrene. The liver from this duck was normal when removed 18 days after the last dose of methylcholanthrene had been given. It is of interest to note that Duck 1038 was given the same amount of methylcholanthrene and sacrificed at the same time as Duck 1027 but no methylcholanthrene was found in the liver. Ducks 1034 and 1041 were sacrificed 18 hours after the last capsule of methylcholanthrene was given orally and

neither showed any of the carcinogen in the liver. There was no methylcholanthrene found in the five livers that showed extensive cirrhotic changes (Table 1).

In view of the possibility that methylcholanthrene might be metabolized in the skin, intestinal tract and trachea, these specific tissues were examined at intervals following local administration of methylcholanthrene to each of these three specific sites. The skin beneath the wing on the right side of six ducks was treated daily for five days with 0.5 ml. of the 0.25 per cent solution of methylcholanthrene. Fifteen minutes after the last application of the carcinogen, birds 1020 and 1021 were sacrificed and specimens of their skin removed. Twenty-four hours later Ducks 1119 and 1112 from the group were sacrificed. Five days after the last application of methylcholanthrene was applied to the skin, Ducks 1122 and 1118 were sacrificed and specimens of the skin removed. Methylcholanthrene was found only in the skin of the two Ducks, 1020 and 1021, sacrificed 15 minutes after the

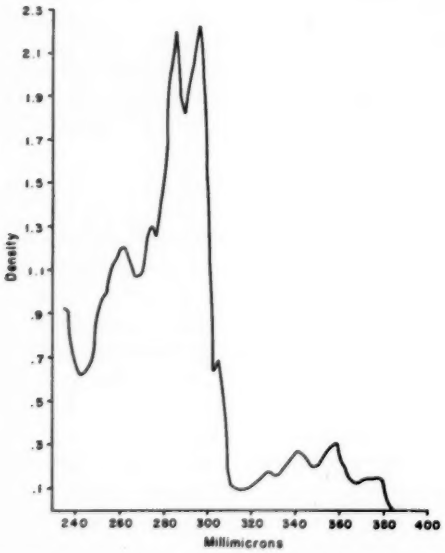

FIG. I. Ultraviolet absorption curve of methylcholanthrene in methanol. There are two peaks that occur at approximately 285 and 295. This is the control.

last application of the carcinogen (Figure III). No methylcholanthrene was demonstrated in the skin of the two birds when sacrificed either 24 hours or 5 days after the last local application of the carcinogen.

Two Ducks, 1151 and 1145, had 0.5 ml. of methylcholanthrene in mineral oil put into the trachea daily for 15 times through the external larynx. Duck 1151 died immediately following the 15th treatment and the trachea was immediately removed. The second bird, 1145, was given 17 treatments and was killed 24 hours after the last. The trachea from both of these birds showed a necrotic membrane covering the inner surfaces.

TABLE 1

Methylcholanthrene in the liver of ducks with cirrhosis

Ducks	Cirrhosis*	Methyl† Cholanthrene in Liver	Remarks
143	+	0	M.C.‡ in acetone applied to skin daily for 45 days. Sacrificed one year later.
457	+	0	M.C. in acetone applied to skin daily for 30 days. Sacrificed 10 months later.
480	+	0	Same as D 457. Sacrificed 1 year later.
904	+	0	8 applications of carcinogen to skin. Died 24 hours after the last.
913	0	0	M.C. pellets put into brain, subcutaneous tissue and muscle. Sacrificed 8 months later.
914	+	0	Same as D. 913. Sacrificed one year later.
915	0	0	Same as D. 913.
1027	0	+	A 50 mg. capsule of M.C. given twice daily for 2 days. Last given 18 days before duck sacrificed.
1037	0	0	Same as D. 1027. Last dose given 4 days before duck sacrificed.
1038	0	0	Same as D. 1027. Last dose given 18 days before duck sacrificed.
1034	0	0	Three 50 mg. capsules of M.C. given by mouth within period of 28 hrs. Last given 18 hrs. before duck sacrificed.
1041	0	0	A 50 mg. capsule of M.C. given twice daily for 10 days. Last given 18 hrs. before duck sacrificed.
1044	0	0	10 mg. of M.C. crystals blown into trachea daily for 30 days. Last given 45 days before duck sacrificed.
1045	0	0	Same as D. 1044. Last given 4 days before duck sacrificed.
1048	0	0	Same as D. 1044. Last given 4 days before duck sacrificed.
1050	0	0	Same as D. 1044. Last given 4 days before duck sacrificed.

* Cirrhosis: + present, 0 absent.
† Methylcholanthrene in extract of liver as shown by spectrophotometer: + present, 0 absent.
‡ M.C. abbreviation for methylcholanthrene.

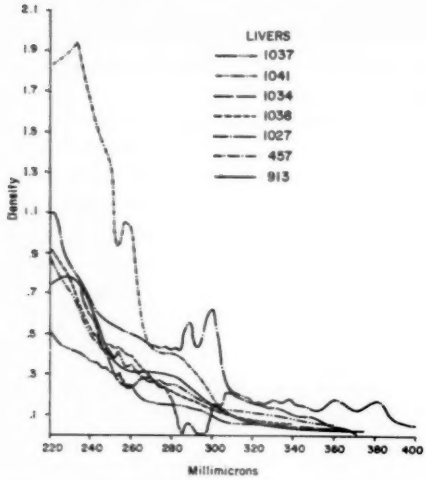

FIG. II. Ultraviolet absorption curve of the benzene extracts of the livers from 7 ducks previously given methylcholanthrene (see Table 1). Only Duck 1027 shows a curve that is consistent with that produced by methylcholanthrene.

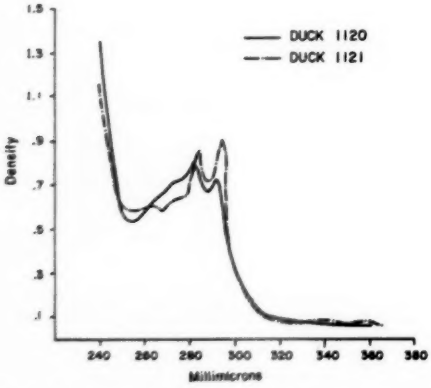

FIG. III. Ultraviolet absorption curve of the benzene extracts of the skin from 2 ducks treated locally with methylcholanthrene and sacrificed 15 minutes after the last application. Both curves are consistent with that of methylcholanthrene.

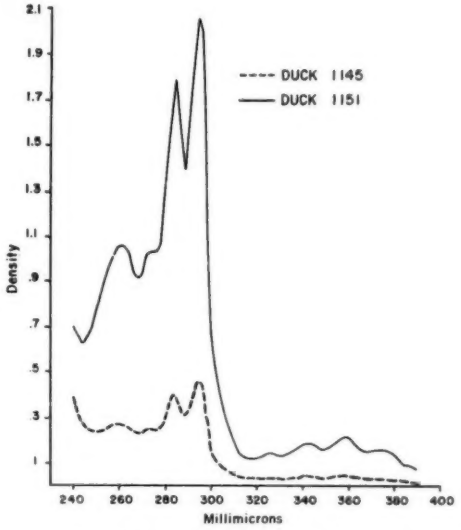

FIG. IV. Ultraviolet absorption curves of the benzene extracts of the trachea from Duck 1151 treated 15 times with 0.5 ml. of methylcholanthrene in mineral oil. Death occurred immediately after the last treatment. Duck 1145 was similarly treated for 17 times and sacrificed 24 hours after the last. Both curves are consistent with that of methylcholanthrene.

Methylcholanthrene was demonstrated spectrophotometrically in the respiratory tract of both of these specimens (Fig. IV).

The feces were collected from Duck 1041 during the time that he was being given capsules containing 50 mg. of methylcholanthrene orally twice daily. The feces were positive for methylcholanthrene.

Discussion

It is obvious from these experiments that methylcholanthrene, as such, is either rapidly removed or rapidly metabolized when put on the skin or into the trachea of white Pekin ducks. Methylcholanthrene was demonstrated in the skin of two ducks in which the specimens of skin were removed 15 minutes after the last application of the carcinogen. However, two birds similarly treated and the skin removed 24 hours later and two other ducks sacrificed five days later were all negative for methylcholanthrene. Methylcholanthrene could be demonstrated spectro-

photometrically in the trachea immediately after it was put through the larynx. However, examination of the trachea with ultra violet light in other experiments has shown no florescent material consistent with methylcholanthrene 24 hours after this carcinogen in mineral oil was put into the trachea. A study of the trachea following the administration of methylcholanthrene and mineral oil with ultra violet light is now being made and will be reported subsequently.

The observations of Beck and Peacock (1940), Hieger (1936), and Simpson and Cramer (1943) have shown that carcinogens usually are rapidly removed from the skin of mice. Beck and Peacock (1940) observed that 3,4-benzpyrene dissolved in benzene disappeared from the skin of mice four days following a single application as determined by an ultraviolet fluorescence test. Hieger (1936) was able to detect fluorescence on the skin of mice three weeks after they were last painted with 1,2,5,6-dibenzanthracene dissolved in benzene. Simpson and Cramer (1943) observed that the fluorescence in the skin due to methylcholanthrene in benzene disappeared from the skin of mice in 6 to 10 days. During this time the majority of the carcinogen was found in the keratin and sebaceous glands. We have observed that the duck likewise shows a rapid decrease of methylcholanthrene from the skin and trachea. Apparently the rate of disappearance from the skin may vary with the host. Furthermore, we have observed that methylcholanthrene persists in the trachea for a much longer time in the presence of an exudate on the surface than it does on the surface of a normal trachea.

The failure to demonstrate methylcholanthrene in the liver of 15 in a group of 16 ducks would suggest that methylcholanthrene, as such, is most likely not absorbed from either the skin or the intestinal tract and transmitted, as such, to the liver. Lorenz and Shimkin (1941–42) found that methylcholanthrene, dispersed in horse serum saturated with cholesterol to equal 1.0 mg. of the hydrocarbon per 1.0 cc. of the vehicle and injected intravenously into mice, diminished within four days to one-tenth of its original value. If a similar rate of disappearance should occur in the liver of the duck, we would not expect to demonstrate it in the cirrhotic livers. Two ducks were sacrificed 18 hours after 50 mg. of methylcholanthrene were given orally and none of the carcinogen was demonstrated in the liver.

One may assume that a metabolite from methylcholanthrene is formed either at the site of local application or elsewhere in the body and this hypothetical substance could have injured the hepatic cells, accounting for the cirrhotic lesion previously reported (Rigdon, 1955). In support of this opinion, it may be recalled that Dobriner, Rhoads and Lavin (1939) found a phenol-like substance in the excreta of rabbits following the subcutaneous injection of 1,2,5,6-dibenzanthracene and suggested that certain animals may have the ability to convert certain carcinogens from a neutral to a phenolic compound.

Summary

White Pekin ducks after receiving methylcholanthrene have been found to have a peculiar type of cirrhosis. None of this carcinogen could be demonstrated in these livers by spectrophotometric techniques. This carcinogen rapidly disappears from the skin following local applications.

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[a] From the Laboratories of Experimental Pathology and Industrial Hygiene, Medical Branch, The University of Texas, Galveston, Texas. Aided by research grants C-1469 (4C) from the National Cancer Institute, of the National Institutes of Health, Public Health Service and The Tobacco Industry Research Committee. Accepted July 30, 1957.

[b] 3-methylcholanthrene M.P. 176°–178° C. Eastman No. 4383.

STUDIES ON ADRENAL ASCORBIC ACID. I. THE FATE OF ADRENAL ASCORBIC ACID

LOTHAR L. SALOMON[a]

The concentrated effort which has been directed at elucidating the physiological role of ascorbic acid within the last three decades has failed to uncover the manner in which this substance prevents scurvy. It may be speculated that the biochemical lesion in scurvy resides in defective hormonal synthesis or activity. Suggestive is the fact that certain endocrine organs are particularly well supplied with ascorbic acid. Not only is this true in species independent of exogenous sources of this vitamin, but also in the few scurvy-liable species, provided they have an adequate intake of ascorbic acid.

Adrenal glands have been a particular target in research. Sayers *et al.* have shown that ACTH (adrenocorticotrophic hormone) depletes them of adrenalcortical cholesterol and ascorbic acid (10). The trophic hormone also enhances adrenalcortical synthesis of steroid hormones. Hence, the loss of cholesterol from the glands has found a ready explanation because, as first shown by Bloch (1), this sterol can serve as a precursor of steroid hormones. The concomitant loss of adrenalcortical ascorbic acid has proved to be a more refractory problem.

By analogy with the role of cholesterol as a precursor, Lowenstein and Zwemer have proposed the existence of steroid-ascorbic acid conjugates (4, 14), based on their isolation of material from adrenal venous plasma possessing lipoid properties, and apparently also containing ascorbic acid. Other investigators have not supported this idea (12). Since the discovery was made that the activity of the adrenal cortex bears no relation to its level of ascorbic acid in the chick (2) and quail (13), it has been variously suggested that the depletion of ascorbic acid in other species might bear only an incidental relationship to glandular activity. Thus, it has been considered that changes in permeability may lead to a depletion of ascorbic acid from the adrenal cortex (11).

The fate of ascorbic acid, leaving the glands consequential to the action of ACTH, might shed some light on this problem. It

has been shown that the amount of ascorbic acid disappearing from the glands is balanced by an equal amount of material appearing in adrenal venous plasma, which may have been ascorbic acid, or dehydroascorbic acid or diketogulonic acid (11). In this question, however, resides the crux of the problem. For if ascorbic acid left the glands as such, without chemical transformation, it may well have left simply as a consequence of some non-specific effect. But if the loss involved a chemical change in the ascorbic acid molecule, it would appear likely that the depletion promotes or controls some glandular function.

The present study was concerned with the fate of adrenal ascorbic acid. It was shown that in adrenal venous plasma of hypophysectomized rats there was present, after administration of ACTH, a significant amount of material which was derived from, but no longer identical with ascorbic acid. This finding indicates that the depletion of adrenal ascorbic acid by ACTH is not a chance occurrence, resulting from some event unrelated to the function of ascorbic acid. It appears likely that the chemical changes which ascorbic acid undergoes during this process may be coupled to some cellular component in such a way as to promote or direct adrenalcortical steroidogenesis.

Methods and Materials

Hypophysectomized rats used in these experiments were males of the Sprague-Dawley strain, weighing 300 ± 50 gm., obtained from Hormone Assay Laboratories, Chicago, Illinois. Normal rats, obtained from the local colony, were descendants of the Sprague-Dawley strain, weighing 300 ± 30 gm. Animals were maintained on Purina laboratory chow and distilled water, *ad libitum*. Hypophysectomized animals were used 3 to 4 days postoperatively.

Ascorbic-1-C^{14} acid was synthesized as previously described (9), with a specific activity of 3.4 μc per mg. It was administered by intraperitoneal injection (dissolved in water, in doses of approximately 7 μc) 12 hours before blood samples or adrenal glands were obtained.

Cannulation Procedure. Prior to the operation, 5 mg. of sodium pentobarbital per 100 gm. of body weight was administered intraperitoneally. Although this proved excessive for a few animals, such a dose was generally necessary to insure continued anesthesia throughout the period of cannulation. Sodium pentobarbital has no effect on adrenal ascorbic acid.[b]

One hundred fifty U.S.P. units of heparin (sodium salt) was injected into the right femoral vein, the left renal vein was ligated, 8 I. U. of ACTH was administered through the left femoral vein, a cannula was inserted into the adrenal vein in such a way as to minimize dilution of adrenal venous blood by blood from other sources, and the left renal vein was then ligated at the

juncture with the vena cava. The entire procedure occupied less than 10 minutes. Systemic blood was obtained by heart puncture at the conclusion of the cannulation process.

Identification and Measurement of Radioactivity. The blood was immediately centrifuged, and the plasma layer added to trichloroacetic acid solution containing carrier ascorbic acid. The amount of carrier varied from 100–175 mg., depending upon the amount of plasma which was anticipated. Greater quantities of carrier cause excessive dilution of the radioactivity.

In the case of adrenal glands, the tissues were ground in a glass homogenizer with trichloroacetic acid, containing approximately 250 mg. of carrier ascorbic acid. Since adrenal glands always had a relatively high level of radioactivity under the conditions of these experiments, such an amount of carrier resulted in high counting rates in the final samples.

Aliquots of the trichloroacetic acid preparations were converted to CO_2 by the wet washing method (3, 6) to give samples of $BaCO_3$; the latter were counted in a windowless flow-counter. After suitable corrections for background, self-absorption and volume, the total radioactivity of the trichloroacetic acid extract could be calculated. Radioactivity was measured under closely controlled conditions to maintain constancy of area, backscattering and geometry, and to avoid the interference of humidity. Equipment was calibrated with samples prepared from National Bureau of Standards Beta-Ray Standard.

From the remainder of the trichloroacetic acid extract, ascorbic acid was isolated, substantially as previously described (9), except that the use of Amberlite IR-4B acetate for the removal of ascorbic acid and the elution with 1 *N* formic acid, was found to be more advantageous. Under these circumstances, it was frequently unnecessary to remove trichloroacetic acid at all prior to passage of the extract through the resin column, since it was not eluted together with ascorbic acid. Only when the concentration of trichloroacetic acid was so high as to potentially saturate or exceed the capacity of the resin, was a portion of the protein precipitant removed by preliminary extraction with diethyl ether. Upon removal of the solvent from those fractions containing ascorbic acid, crystals were readily obtained. Recrystallization, and testing for chemical and radioactive purity followed established procedures (8). Accurately known amounts of the material isolated in this way were converted to $BaCO_3$ and the total amount of radioactivity contained in ascorbic acid calculated from the counting data.

Chemical assays for ascorbic acid were carried out by a scaled-up version of the micro-method of Lowry et al. (5).

Results and Discussion

Prior to investigations into the nature of the radioactive material in adrenal venous plasma, it was necessary to ascertain the status of the radioactive material *within the gland* itself. Table 1 shows that essentially all radioactivity in adrenal glands could be isolated as ascorbic acid, whether or not ACTH had been ad-

ministered. Should non-ascorbic acid radioactivity appear in adrenal venous plasma, it must, therefore, constitute a newly-formed product.

When pooled samples of adrenal venous plasma from hypophysectomized rats were collected by cannulation, after administration of ACTH, a considerable fraction of the radioactivity was

TABLE 1

Identity of Radioactivity in Adrenal Glands of Hypophysectomized Rats after Injection of Ascorbic-1-C^{14} Acid

No ACTH Per Cent	ACTH Per Cent
99	99
97	103
101	96

Values represent per cent of radioactivity in trichloroacetic acid extracts of adrenal gland homogenates which could be identified as unchanged ascorbic acid. Within the precision of the method, no radioactive substances other than ascorbic acid appeared to be present under the conditions of these experiments. Pooled adrenal glands of two or three rats were used in each determination.

TABLE 2

Radioactivity and Radioactive Ascorbic Acid in Adrenal Venous and Systemic Plasma after Injection of Ascorbic-1-C^{14} Acid and ACTH

Experiment No.	1	2	3	4
	Radioactivity in Plasma			
	cpm/ml.	cpm/ml.	cpm/ml.	cpm/ml.
Adrenal Venous	2,320	2,880	1,600	7,050
Systemic	1,530	1,910	1,120	1,780*
				2,280
	Radioactivity in Isolated Ascorbic Acid			
	Per Cent	Per Cent	Per Cent	Per Cent
Adrenal Venous	82	80	71	62
Systemic	99	104	92	96*
				98

* Represents specimen obtained prior to administration of ACTH. All others obtained after administration of ACTH.

Data represent radioactivity (in counts per minute per ml.) in adrenal venous and systemic plasma, and the fraction of this radioactivity (in per cent) which could be isolated as unchanged ascorbic acid. Adrenal venous plasma had a consistently higher radioactivity than systemic plasma, indicating the liberation of additional radioactive material from adrenal glands into adrenal venous plasma. However, each value represents the radioactivity of a plasma sample pooled from specimens of several animals. In making pooled samples, maximum obtainable amounts, rather than proportionate amounts from each animal, were used. Consequently, a direct comparison of the radioactivity between systemic and adrenal venous plasma has limited validity.

no longer present as ascorbic acid. Table 2 shows the results of four such experiments, each sample comprising adrenal venous plasma of three rats. Systemic plasma, pooled from the same rats, does not exhibit this characteristic. This is somewhat surprising, since the right gland was left intact, and must have liberated such material into systemic blood. It would appear, therefore, that this non-ascorbic acid radioactivity is either rapidly eliminated (*via* the kidneys, by removal into some other tissue, or by rapid reconversion to ascorbic acid), or that dilution by systemic plasma was great enough to obscure the presence of the material. It may be noted that the method of detection employed would probably fail to show differences of 5 per cent or smaller with any reasonable degree of certainty.

The nature of the radioactive fraction in adrenal venous plasma which is not ascorbic acid, cannot be deduced from these data alone. However, Slusher and Roberts (11) found that ascorbic acid lost from the adrenal glands appeared as ascorbic acid, dehydroascorbic acid, or diketogulonic acid in the venous effluent; the method of assay used by these workers did not allow them to distinguish between these substances. Since the present data tend to eliminate ascorbic acid, dehydroascorbic acid or diketogulonic acid are the remaining possibilities.

It may be concluded that ACTH caused an acceleration of the oxidation of ascorbic acid in the adrenal cortex. In a coupled redox reaction, another cellular component may thereby have become reduced. This could constitute an important step in steroidogenesis. Further, the ability to maintain a concentration gradient of ascorbic acid (which is approximately 500:1 between the glands and plasma) may not be paralleled by an ability to maintain a steep concentration gradient of dehydroascorbic acid or diketogulonic acid. The cause of the disappearance of ascorbic acid from the glands due to the action of ACTH could simply be the absence of a barrier to dehydroascorbic acid.

Were ascorbic acid to flow from the glands merely because of some change incidental to the action of ACTH, but accidental in so far as ascorbic acid were concerned, one would not expect chemical changes to accompany such a process. The events as they apparently occurred indicate that, as has been suggested before, ascorbic acid may promote the synthesis of steroid hormones by leaving the glands, rather than by being there (7).

Slusher and Roberts have reported the occurrence of extra-adrenal ascorbic acid in blood during prolonged cannulation (11). Similar observations have been made in this laboratory. Parallel findings were made in guinea pigs, moribund from the effects of diphtheria toxin (8). Experience has shown this to accompany respiratory distress. It is felt that this may be an essential factor.

From the point of view of the present study, a more relevant observation concerns the effect of trauma to the adrenal glands. In initial experiments, designed to explore proper conditions for cannulation, it was noted that temporary stoppage of circulation through the adrenal glands produced corticotropin-independent release of ascorbic acid. Although this effect was remarkable and totally unexpected, it nevertheless presented a problem in this case. To circumvent this difficulty, ligation of the adrenal vein was avoided, and animals were not used when it appeared that significant interference with adrenal circulation had occurred during cannulation. Figure I shows some pertinent data. It may be noted that, although ligation produced release of ascorbic acid, this effect was enhanced by ACTH, and eliminated by avoidance of ligation. Under the circumstances, the possibility that levels of ascorbic acid in adrenal glands of hypophysectomized rats may be decreased by lowered oxygen tensions, cannot be altogether disregarded. To eliminate as much as possible interference from corticotropin-independent release of ascorbic acid, cannulations were terminated whenever the animal appeared to be in respiratory distress, or when the cannulations appeared not to be proceeding in an entirely satisfactory way. It is believed that equivocal samples were eliminated in this manner.

Summary

In studies with hypophysectomized rats, which had received doses of radioactive ascorbic acid, it was found that all radioactive material within the adrenal glands could be identified as ascorbic acid, both before and after the administration of ACTH.

Following the administration of ACTH, radioactive material appeared in adrenal venous plasma which could no longer be identified as ascorbic acid, although systemic plasma from the same animals contained no radioactive substances other than ascorbic acid. This indicated that ascorbic acid underwent some

chemical transformation prior to its release from the adrenal cortex.

In conjunction with another report (11), this result makes it appear likely that ascorbic acid was converted to dehydroascorbic acid or diketogulonic acid due to the action of ACTH on the adrenal cortex. It is suggested that the subsequent de-

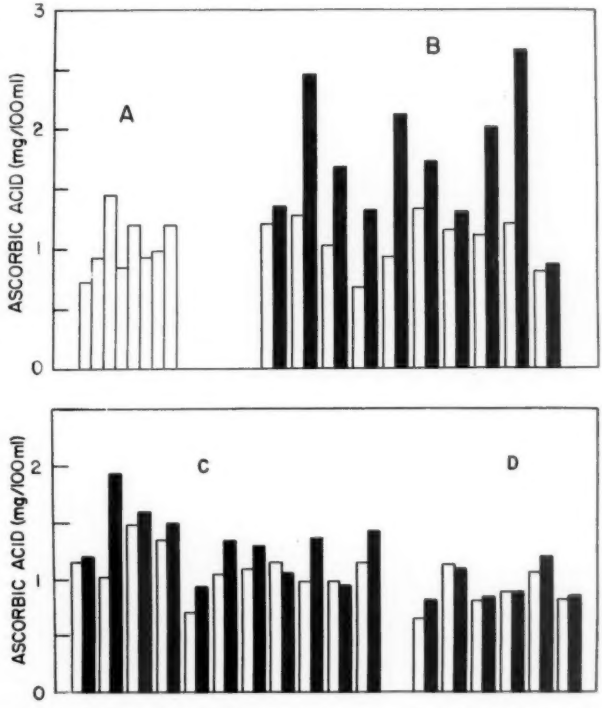

FIG. I (A, B, C, and D). Concentrations of Ascorbic Acid in Adrenal Venous and Systemic Plasma of Hypophysectomized, Cannulated Rats.

The bars in figure I A represent ascorbic acid concentrations found in systemic plasma of 8 hypophysectomized rats; these rats were not cannulated. The bars in figures I B, C, and D contrast ascorbic acid concentrations in adrenal venous plasma (solid bars) with systemic plasma. Each pair of bars represents data from a single rat. Figure I B is based on data from rats in which the adrenal vein was briefly ligated, and which had received ACTH. Figure I C is based on data from rats in which the adrenal vein was briefly ligated, but which did not receive ACTH. Figure I D is based on data from rats in which ligation of the adrenal vein was avoided during the cannulation procedure, and which did not receive ACTH.

pletion of adrenal ascorbic acid resulted from a low adrenal-cortical retentivity for dehydroascorbic acid or diketogulonic acid.

Interference with circulation through the adrenal gland elicited corticotropin-independent release of ascorbic acid into adrenal venous plasma. It is suggested that this, and similar observations, was caused by tissue anoxia.

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[b] In experiments with a limited number of normal rats, it was found that the administration of 15 mg. of sodium pentobarbital did not significantly affect the concentration of adrenal ascorbic acid. Neither the drug itself, nor the manipulation and injection appeared to constitute a stress. 448 mg. of ascorbic acid per 100 gm. of adrenal tissue (400 to 520 mg., 7 animals) was found in untreated rats. In anesthetized rats, 15 minutes after treatment, 436 mg. per 100 gm. of tissue (395 to 489 mg., 4 rats) was found. By contrast, during the first 20 minutes after administration of 8 I. U. of ACTH, adrenal ascorbic acid was depleted by approximately 30 per cent in normal rats (average, 311 mg. per 100 gm. of tissue, 283 to 360 mg., 4 rats).

STUDIES ON ADRENAL ASCORBIC ACID. II. ON ASCORBIC ACID NEOGENESIS IN RAT ADRENAL GLANDS

LOTHAR L. SALOMON[a]

During the course of studies on ascorbic acid metabolism, it became of interest to determine whether rat adrenal glands are capable of synthesizing ascorbic acid. Although it has been shown that the glands cannot be the sole source of ascorbic acid in the rat or dog (10, 11), the degree of contribution from this source to the total arising from all tissues remained in question. An additional point of interest related to the effect of ACTH. This hormone tends to diminish the concentration of adrenal ascorbic acid (8, 9) and possibly exerts some influence on its synthesis.

Studies on the exchange of ascorbic acid between adrenal glands and extracellular fluid yielded data which indicated that ascorbic acid synthesis in adrenal tissue is either totally absent, or quantitatively unimportant both to the animal as a whole, and to the glands themselves. That part of the data which bears on ascorbic acid synthesis in rat adrenal glands is here presented.

Methods and Materials

Male hypophysectomized Sprague-Dawley rats, weighing between 140 and 160 gm., received ascorbic-1-C^{14} acid (7) intraperitoneally in 0.5 ml. of water 5 to 6 days postoperatively.[b] Doses of approximately 3.4 μc (1 mg.) were administered. After the desired periods, as indicated in figure I and table 1, the animals were decapitated, the adrenal glands removed, dissected free of adhering tissue, and weighed. The blood of these rats was collected in chilled beakers containing heparin, immediately centrifuged, and plasma samples removed. The concentrations of ascorbic acid (4) and radioactivity were then determined on trichloroacetic acid extracts of adrenal gland homogenates or plasma. Aliquot portions of this extract were converted to $BaCO_3$, from which samples were prepared for counting in a windowless flow-counter (2, 5). The usual corrections for background, self-absorption, volume differences, and differences in administered radioactivity were made. All samples were analyzed in duplicate, and routinely counted to a probable error of $\pm$ 2 per cent.

The ratio of counts per minute per unit quantity of tissue or plasma to mg. of ascorbic acid per unit quantity of tissue or plasma, gave the specific activity of ascorbic acid. This simple procedure could be applied because it has been

found previously that virtually all systemic plasma and adrenal radioactivity can be identified as unchanged ascorbic acid under the conditions of this experiment (6). The results of these measurements are shown in figure I as average specific activities.

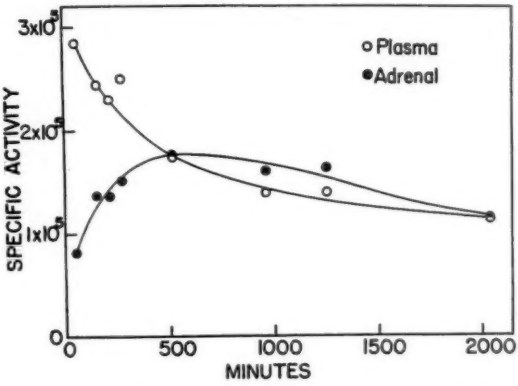

FIG. I. Specific activities of plasma ascorbic acid and adrenal ascorbic acid in hypophysectomized rats. These animals received 1.0 mg. of ascorbic-1-C^{14} acid (3.4 μc), by intraperitoneal injection. Each point represents the average specific activity (in counts per minute per mg. of ascorbic acid) of the number of separate determinations shown by the corresponding values of Table 1.

Results and Discussion

Table 1 shows that both adrenal and plasma ascorbic acid concentrations remained constant during the experimental period. The rate at which ascorbic acid increased in the glands may be represented by

$$(1) \quad dA_{ad}/dt = a + b = K$$

where a is the constant rate at which ascorbic acid was synthesized, and b the constant rate at which it entered from interstitial fluid. Similarly the rate at which adrenal ascorbic acid decreased may be represented by

$$(2) \quad -dA_{ad}/dt = K'$$

Here, K′ may represent a single constant rate, or the summation of several constant rates, according to whether adrenal radioactivity was removed from the glands as one or more molecular

species. Equations *(1)* and *(2)* must balance, because of the existence of a steady state, hence, $K + K' = 0$.

Adrenal radioactivity must originate exclusively in extracellular fluid, unlike ascorbic acid, which might by synthesized *in*

TABLE 1

Concentrations of Plasma Ascorbic Acid after Injection of Ascorbic-1-C^{14} Acid

Time (min.)	50	150	210	270	510	960	1260	2040
	mg. per 100 ml. of plasma							
	1.48	1.01	1.00	0.95	1.15	0.65	0.99	1.08
	1.10	1.19	0.91	0.81	1.01	0.73	0.78	1.31
	0.92	1.17	0.97	0.88	0.90	1.05	0.74	1.18
	2.29	1.20	0.96	0.66	0.97	1.08	0.71	1.07
	2.15		1.16	0.71	1.40	0.79	0.74	0.73
	1.48				1.13	1.06	0.83	0.80
					1.02	1.29	0.77	0.84
						0.77	0.87	
						0.74	0.82	
						0.58		
						0.93		
Average	1.58	1.14	1.00	0.80	1.08	0.88	0.81	1.00

Concentrations of Adrenal Ascorbic Acid after Injection of Ascorbic-1-C^{14} Acid

	mg. per 100 gm. of tissue							
	303	346(4)*	320	321(3)*	346	276	333	313(4)*
	257		295	318	300	289	249	379
	330		324	223	299	294	289(4)*	331
	334		300		327(4)*	320(2)*	317	335
	353		339			277(2)*		
	353					287		
						335		
						360		
						256		
Average	322	346	316	301	322	299	294	328

* Pooled sample, composed of the number of pairs of adrenal glands shown in parentheses. In computing averages including these values, weighting was used.

Each value represents the concentration of ascorbic acid in plasma or adrenal glands of a single hypophysectomized rat, unless marked by an asterisk, at the indicated time after injection of 1.0 mg. (3.4 μc) of radioactive ascorbic acid. It may be noted that, except for some effect due to the injection at the 50 minute period, which is restricted to plasma alone, the concentrations of ascorbic acid remained constant within the limits of individual differences. The equation *(3)* is independent of the plasma concentration. However, equality of inflow and outward flow of ascorbic acid, and the constancy of this flow, was assumed, and this appears to be justified by these data. While changes in concentration of adrenal ascorbic acid do occur after hypophysectomy, the postoperative period was chosen to be sufficiently extended for a reasonably constant level of concentration to be attained.

situ. The radioactivity associated with b mg. of ascorbic acid entering the gland per unit time is given by br_p, where r_p is the specific activity of plasma (and substantially, interstitial) ascorbic acid. The processes which tend to decrease adrenal radioactivity may be stated as $K'r_{ad}$, where r_{ad} is the specific activity of adrenal ascorbic acid. The net rate of change in adrenal radioactivity must, therefore, be

$$(3)\quad dR_{ad}/dt = br_p + K'r_{ad} = (K - a)r_p + K'r_{ad}$$

Experimentally, it was found that a maximum in the specific activity-time curve of adrenal ascorbic acid occurred at about 8.5 hours after ascorbic—1—C^{14} acid administration and that, at the same time, the values of r_{ad} and r_p became identical. At this point, $dR/dt = 0$, and $K - a = -K' = K$, which is not possible, *unless* $a = 0$. Thus, the specific activity of adrenal ascorbic acid can become identical with that of plasma ascorbic acid only when there is no synthesis by adrenal tissue. Experimental results here reported indicate that, in fact, such synthesis either does not occur at all, or only at a negligible rate.

The radioactivity data show that ultimately adrenal ascorbic acid equilibrates completely with plasma ascorbic acid. Therefore, adrenal ascorbic acid apparently arises by removal from extracellular fluid, even in the rat, an animal which is independent of exogenous sources of vitamin C. Following the depletion induced by ACTH, the adrenal ascorbic acid becomes more rapidly reconstituted in the rat than in the guinea pig (3). Rats are independent of exogenous sources of ascorbic acid, but guinea pigs are not. Nevertheless, the evidence shows this to be certainly not due to synthesis of ascorbic acid in the adrenal glands of the rat. Despite the high concentrations found in adrenal glands, adrenal tissue seems not to supply ascorbic acid to the rat as a whole, or to itself. The ability of the gland to store the vitamin, however, becomes all the more remarkable in view of this. Liver tissue of rats has been reported to be capable of synthesizing ascorbic acid (1). It is interesting to find that the ability to synthesize ascorbic acid appears not to be uniformly shared by all of the soft tissues of the rat, and that tissues relatively low in ascorbate content may synthesize the vitamin, while others do not, in spite of their high ascorbate levels.

It should be emphasized that it is not intended to prove a com-

plete absence of ascorbic acid synthesis. The experimental methods were such as to make it difficult to pinpoint the maximum in specific activity of adrenal ascorbic acid very accurately except to show that it occurred near or at the point of intersection between the two curves. However, should a low rate of synthesis occur, it cannot be of importance even to the gland itself. Since ascorbic acid in the glands is in continuous and facile exchange with extracellular ascorbic acid, as shown by the radioactivity data, it cannot be dependent on synthesis *in situ* to supply or maintain its required ascorbic acid. Strictly speaking, the above data apply to hypophysectomized rats only. Preliminary data, obtained in connection with a related study to be published separately, indicate that the applicability extends to intact rats as well.

Summary

The adrenal glands of rats contain a very high concentration of ascorbic acid; however, there is evidence that biosynthesis *in situ* does not contribute significant amounts. Hypophysectomized rats received ascorbic —1—C^{14} acid intraperitoneally, and the specific activity of adrenal and plasma ascorbic acid was determined periodically thereafter. Analysis of the data showed that the occurrence of a maximum in the specific activity of adrenal ascorbic acid, coincident with equality with the specific activity of plasma ascorbic acid, can be reasonably explained only on the basis that adrenal synthesis of ascorbic acid is either negligible or non-existent.

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[a] From the Department of Biochemistry and Nutrition, The University of Texas Medical Branch, Galveston, Texas. This research was supported in part by funds provided under contract AF 18(600)–638 with the School of Aviation Medicine, USAF, Randolph Air Force Base, Texas, and by the American Medical Association. Accepted for publication August 27, 1957.

[b] Approximately 20 per cent of the animals were checked over a period of not less than 1 month for weight gain and sexual maturation; completeness of hypophysectomy was indicated in all. Post-mortem examinations were kindly made on a number of rats by Dr. J. C. Finerty of the Department of Anatomy, with similar results. The hypophysectomized rats were purchased from Hormone Assay Laboratories, Chicago, Illinois.

CALLING ATTENTION TO

Items of Possible Interest to Our Friends

July–August 1957

Calling Attention To: Items of possible interest to friends of Chauncey D. Leake *Light Hot-weather Reading:* F. Bello tries to answer the transquilizer questions (*Fortune* 55/162/May, 57). K Clark covers ***The Nude: A Study in Ideal Form*** (Pantheon Books, NY, 57, 512pp, $7.5). JK Donohue sees thru ***The Baffling Eyes of Youth*** (Asso Press, NY, 57, 228pp, $3.5). AH Dupree studies *Science in the Federal Government: A History of Policies and Activities to 1940* (Harvard Univ Press, Cambridge, Mass, 57, 360pp, $7.5). L Klein considers *Aspects of River Pollution* (Academic Press, NY, 57, 621pp, $14.5). R & G Laubin fascinate in ***The Indian Tipi: Its History, Construction & Use*** (Univ Oklahoma Press, Norman, 57, 220pp, $4). J Maritain analyzes *Creative Intuition in Art & Poetry* (Pantheon Books, NY, 57, 423pp, $6.5). JA Michener & AG Day expose *Rascals in Paradise* (Random House, NY, 57, 322pp, $4.75). E Pawel tells of revolt of an organization man ***From the Dark Tower*** (Macmillan, NY, 245pp, $4). DM Roberts proposes ***Partners with Youth*** (Asso. Press, NY, 57, 211pp, $3.5). HS Schwartz tries a hand at *Home Care for the Emotionally Ill* (Sessions Publications, NY, 57, 320pp, $5). J Thurber as ever amuses in ***The Wonderful O*** (Simon & Schuster, NY, 57, 72pp, $3.5). B Ward looks wisely at ***The Interplay of East & West*** (Norton, NY, 57, 168pp, $3.5). A Windomuth discusses health team relations in ***The Nurse & the Outpatient Department*** (Macmillan, NY, 580pp, $9).

Clinical: HK Beecher really analyses the measurement of pain (***Pharmacol Rev*** 9/59/57). A Cournand describes control of pulmonary circulation in humans (*Science* 125/1231/June 21/57). FF Foldes surveys *Muscle Relaxants in Anesthesia* (CC Thomas, Springfield, Ill, 57/224pp, $5.5). A Gronwall discusses *Dextran & Its Use in Colloidal Infusion Solutions* (Academic Press, NY, 57, 156pp, $4). H Hoagland edits Arden House Conference on *Hormones, Brain Function & Behavior* (Academic Press, NY, 57, 257pp, $7). RR Ichelson shows Spirochaeta myelophthera in spinal fluid of multiple sclerosis patients (Proc Soc Exp Biol Med 95/57/57). WS Middleton has VA Dept Med & Surg prepare *Medical Research in the Veterans Administration* (US Govt Printing Office, Washington DC, 57, 945pp, $4). R Pratt ably reviews use of antibiotics in elderly patients (*Geriatrics* 11/341/57). DW Richards describes contributions to medicine from right heart catheterization (*Science* 125/1181/June 14/57). FM Strong heads committee reporting on mortality statistics on smoking and health (*Science* 125/1129/June 7/57).

Preclinical: CS Bachofer finds activation of nerves by X-rays (*Science* 125/1140/June 7/57). J Brachet offers *Biochemical Cytology* (Academic Press, NY, 57, 480pp, $8.8). EJ Conway considers significance of relative concentrations of K & Na ions in muscle (*Physiol Rev* 37/88/57). RC Crofts & HA Meincke find hypophysectomized animals show enemia as reflection of de-

creased oxygen need (*Proc Soc Exp Biol Med* 95/127/57). GP Fulton & Co survey humoral aspects of tissue mast cells (*Physiol Rev* 37/221/57). CO Hebb reviews biochemical evidence for neural function of acetylcholine (*ibid* p 196). HS Heller edits important Colton Research Society Symposium on *The Neurohypophysis* (Academic Press, NY, 57, 275pp, $9.5). MH Knisely & Co summarize knowledge of hepatic sphincters (*Science* 125/1023/May 24/57). GB Koelle demonstrates reactivation of acetylcholinesterase (*ibid* p 1195/June 14/57). SR Korey & JI Nurnberger edit *Neuro-Chemistry* (PB Hoeber, NY, 57, 244pp, $7). L Lasagna & WP McCann find barbitals, the promazines & reserpine reduce amphetamine toxicity in group-stressed animals (*Science* 125/1241/June 21/57). HS Mason discusses mechanisms of oxygen metabolism (*ibid* p 1185/June 14/57). RT Meryman reviews tissue freezing & local cold injury (*Physiol Rev* 37/233/57). W. Pigman well edits *The Carbohydrates: Chemistry, Biochemistry & Physiology* (Academic Press, NY, 57, 930pp, $18). DP Purpura & Co show gamma aminobutyric acid blocks excitatory synapses in brain (*Science* 125/1200/June 14/57). ER Ramey & MS Goldstein survey relations of adrenal cortex and sympathetic nervous system (*Physiol Rev* 37/155/57). SI Rapaport & SB Ames find erythrocyte hemolysate has antiheparin action (*Proc Soc Exp Biol Med* 95/153/57). RL Sinsheimer takes 1st steps toward a genetic chemistry (*Science* 125/1123/June 7/57). H Waelsch edits *Ultrastructure & Cellular Chemistry of Neural Tissue* (PB Hoeber, NY, 57, 230pp, $7).

C. D. Leake

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